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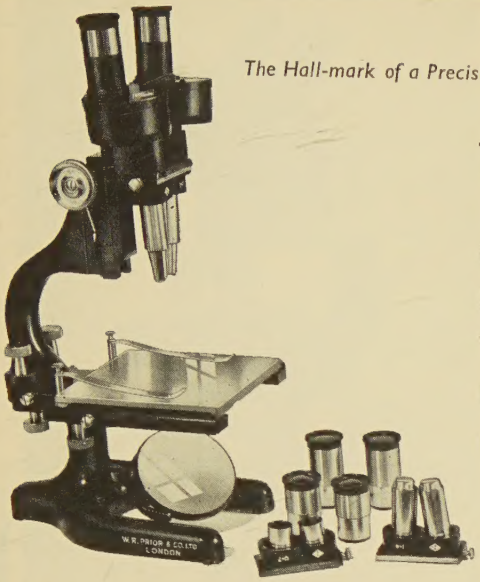

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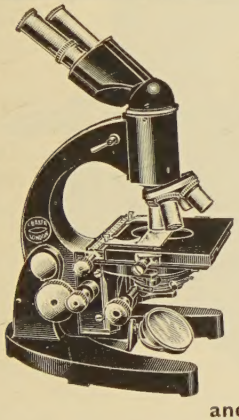

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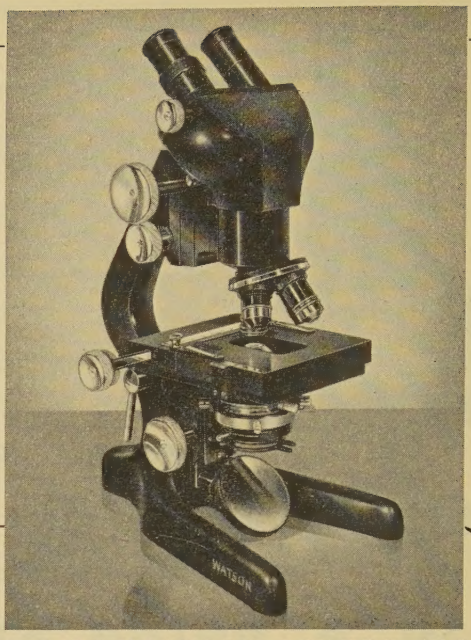

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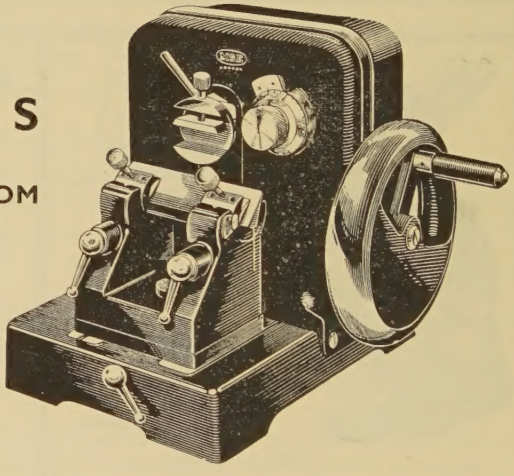

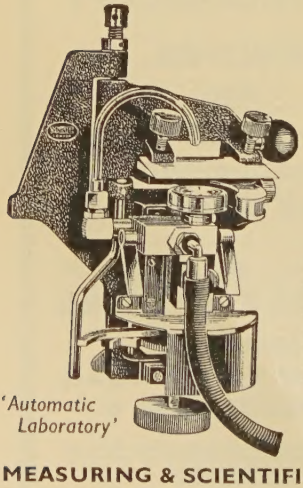

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The Blood-system in the Serpulimorpha (Annelida, Polychaeta)

I. The Anatomy of the Blood-system in the Serpulidae

By JEAN HANSON

(*From the Department of Zoology, Bedford College, University of London; now at the M.R.C. Biophysics Research Unit, King's College, Strand, London*)

SUMMARY

1. An account will be given of the anatomy of the blood-system of *Pomatoceros triqueter*, and of comparative observations on the following serpulids: *Serpula vermicularis*, *S. lo-biancoi*, *Hydroides norvegica*, *Vermiliopsis infundibulum*, *Salmacina incrustans*, *Protula intestinum*, *P. tubularia*, *Apomatus ampulliferus*, *A. similis*, *Spirorbis militaris*, and *S. corrugatus*.

2. In all species there is a central blood-system of large vessels in which blood circulates in the usual manner, and a peripheral system of small blind-ending vessels which are alternately full and empty, receiving their blood from the central system, then returning it along the same channels to the central system.

3. The central blood-system is as follows: Blood moves from the tip of the abdomen to the front of the thorax through a sinus enveloping the alimentary canal. Anteriorly it passes through dorsal, transverse, and circum-oesophageal vessels to a ventral vessel in which it moves backwards to the tip of the abdomen. A pair of ring vessels connects the ventral vessel with the sinus at the posterior end of each segment.

4. The anterior end of the dorsal vessel in *Pomatoceros triqueter*, *Serpula vermicularis*, *Hydroides norvegica*, and *Vermiliopsis infundibulum* is surrounded by a sphincter muscle of unknown function, and contains a muscular valve which probably obstructs the back-flow of blood from the transverse vessel. *Protula intestinum* possesses the valve but lacks the sphincter. *Salmacina incrustans* and *Spirorbis militaris* have neither valve nor sphincter.

5. The peripheral blood-system has the following components: the branchial vessels with branches in the crown; the vessels of the collar and lips; the peri-oesophageal plexuses; the trans-septal vessels supplying the body-wall, parapodia, and thoracic membrane.

6. In *Pomatoceros triqueter* the opercular vessel is spirally coiled, but in other serpulids it is characteristically branched.

7. When *Pomatoceros* withdraws into its tube, movement of blood in the crown ceases. The operculum is therefore not used as a special respiratory organ when the crown is retracted.

8. The oesophagus of *Pomatoceros* is surrounded by two independent blind-ending vascular plexuses, an outer plexus communicating with the gut sinus and an inner plexus with the circum-oesophageal vessels. *Serpula vermicularis* is probably the same. *Hydroides norvegica* and *Protula intestinum* lack the outer plexus. *Salmacina* and *Spirorbis* have neither plexus.

9. The body-wall of each segment derives its blood-supply from trans-septal branches of the ring vessels of the preceding segment. In *Salmacina* and *Spirorbis* these trans-septal vessels are unbranched. In larger serpulids they have numerous

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branches under the epidermis, in the parapodia, and in some cases on the coelomic surface of the body-wall. Branches of the thoracic trans-septal vessels supply the thoracic membrane. In all species except *Salmacina, Spirorbis,* and *Protula intestinum* the thoracic trans-septal vessels end ventrally in two superficial ventro-lateral longitudinal vessels which communicate either with the circum-oesophageal vessels or with the ventral vessel. In *P. intestinum* the thoracic trans-septal vessels enter the ventral vessel directly. The pattern of superficial vessels on the ventral surface of the thorax is useful for identifying specimens.

10. Lateral vessels, such as are found in *Sabella,* are absent in all serpulids.

Contents

Introduction

THIS paper is the first of four which will deal with the anatomy and histology of the blood-system in the Serpulidae and Sabellidae. This research was undertaken as part of a series of investigations by Professor H. Munro Fox and workers in his laboratory on serpulids and sabellids which, together with chlorhaemids, are the only animals definitely known to possess the blood pigment chlorocruorin. The chemical and physical properties of chlorocruorin have been studied by Fox (1924, 1926, 1932, 1934, 1946) and Roche and Fox (1933), the occurrence of chlorocruorin in the Serpulimorpha by Fox (1949), and the respiration and blood circulation of sabellids by Fox (1933, 1938) and R. F. Ewer and Fox (1940). D. W. Ewer (1941) has described the anatomy of the blood-system of *Sabella* and has made preliminary observations on its histology.

Previous Work

A general account of the anatomy of a typical serpulid, *Pomatoceros triqueter*, has been published by Thomas (1940). The body consists of a prostomium bearing the branchial crown, a thorax of several segments of which the first is the peristomium, and an abdomen of many segments. The branchial crown consists of filaments which bear pinnules; one of the filaments is modified as a peduncle carrying an operculum at its tip. At the anterior end of the thorax is a collar; a thoracic membrane extends along each side of the thorax dorsal to the parapodia.

Thomas (1940) has given the most recent account of the blood-system of a serpulid, *Pomatoceros triqueter*. Earlier descriptions were published by Huxley (1855—*Salmacina dysteri*), Claparède (1868–70—*Serpula vermicularis*; 1873—serpulids in general), Haswell (1885—*Hydroides elegans* and *Pomatoceros elaphus*), Jaquet (1886—*Protula intestinum*), Meyer (1888—*Protula*

tubularia), Malaquin (1901—*Salmacina dysteri*), zur Loye (1908—*Spirorbis borealis*), Lee (1912—*Serpula vermicularis*, *Hydroides norvegica*, *Vermiliopsis infundibulum*, *Pomatoceros triqueter*, *Salmacina dysteri*, and *Protula intestinum*), Woskressensky (1924—*Spirorbis militaris*), and Faulkner (1930—*Filograna implexa* and *Salmacina dysteri*). The most important of these papers are those of Meyer, Lee, Faulkner, and Thomas. The work of Jaquet and Meyer was reviewed by Fuchs (1907), and that of Haswell by McIntosh (1918).

The main features already known of the serpulid blood-system can be summarized as follows. A sinus envelops the alimentary canal from the tip of the abdomen to the junction of the stomach and oesophagus just behind the peristomium. Here the sinus leads into a short dorsal vessel connected by circum-oesophageal vessels with a ventral vessel, which extends back along the whole length of the body and communicates with the gut sinus by segmentally arranged ring vessels. Meyer (1888) and Lee (1912) found that the first two pairs of ring vessels are branches of the circum-oesophageal vessels, the first pair leading to the dorsal vessel, and the second pair to the gut sinus; but according to all other observers the first ring vessels lead from the ventral vessel to the sinus. Meyer (1888), Lee (1912), and Thomas (1940) found a lateral vessel connecting the ring vessels on each side of the body. A peri-oesophageal vascular plexus, leading out of the sinus, was described by Claparède (1873), Haswell (1885), and Thomas (1940). Two branchial vessels leave the circum-oesophageal vessels and branch into single blind-ending vessels in each filament and pinnule. The vessels in the collar and thoracic membrane are also blind-ending. The collar vessels arise from the circum-oesophageal vessels. The vessels in the thoracic membrane are branches of ring vessels; according to Thomas (1940) the first pair of thoracic membrane vessels are branches of the circum-oesophageal vessels. Little is known about the blood-supply of the body-wall, parapodia, and intersegmental septa; branches of the ring vessels lead to these structures. According to Faulkner (1930) *Filograna* and *Salmacina* are in many ways different from other serpulids. Her work will be discussed later (p. 125).

Material and Methods

The following species have been used: *Serpula vermicularis* L., *S. lo-biancoi* Rioja, *Hydroides norvegica* (Gunnerus), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Salmacina incrustans* Claparède, *Protula intestinum* (Lamarck), *P. tubularia* (Montagu), *Apomatus ampulliferus* Philippi, *A. similis* Marion and Bobretzky, *Spirorbis corrugatus* (Montagu), and *S. militaris* (Claparède). *Pomatoceros* was obtained from Plymouth, and the other species were studied at Naples. *Serpula lo-biancoi*, previously recorded only from the Atlantic coast of Spain, was found at Naples for the first time in 1947 by Professor H. Munro Fox, who lent me his specimens.

Observations were made on living worms, on benzidine preparations (see below), and on serially sectioned specimens. The whole of the blood-system in *Salmacina* and *Spirorbis* and the superficial vessels of the larger species can

be studied in whole living specimens. Serial sections are needed for tracing the internal vessels in species of a medium size. Most parts of the blood-system of the largest species, *Protula intestinum*, can be studied in living specimens dissected under a binocular dissecting microscope.

Like haemoglobin, chlorocruorin is a peroxidase (Lankester, 1869). In the presence of hydrogen peroxide and benzidine, a blue-black oxidation product of benzidine is deposited in the blood-vessels. This reaction has previously been used by Prenant (1921), Faulkner (1930), and Ewer (1941) for tracing the course of small vessels in polychaetes. I have employed, with equal success, the methods of Slonimsky (1927), Faulkner and Ziegler (1945). It is necessary to use thin specimens. The vessels of the body-wall of medium-sized species, such as *Pomatoceros*, are well demonstrated in preparations made by bisecting the worm longitudinally and removing the alimentary canal. Stained specimens were fixed in 70 per cent. alcohol acidified with a trace of acetic acid to control decolorization. They were then transferred to benzyl alcohol, and subsequently to Canada balsam.

The following fixatives were found to be satisfactory for serially sectioned material: 4 per cent. formaldehyde in sea-water, Duboscq-Brasil, Heidenhain's 'Susa', Zenker-acetic, and Zenker-formol. Most of the sections were stained with 'Azan'.

Observations

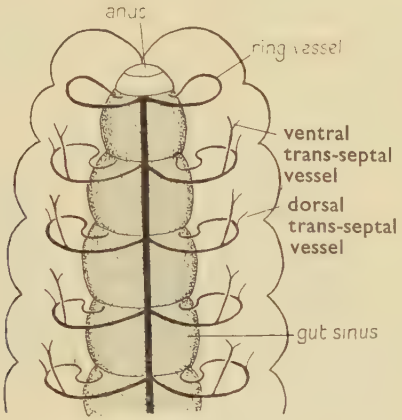
The serpulid blood-system is unusual because all the smaller vessels end blindly and are alternately empty and full of blood. They receive blood from the larger vessels, then the direction of flow is reversed and all the blood returns to the larger vessels. The movement of blood in these small vessels can be watched in the branchial crown, collar, and thoracic membrane. These blind-ending vessels constitute the *peripheral blood-system*, and the larger vessels belong to the *central blood-system*, in which the blood circulates in the usual manner. As in most invertebrate animals it flows forwards dorsally and backwards ventrally. Except at the front of the thorax, a sinus surrounding the alimentary canal takes the place of a dorsal vessel. The blood is moved forwards along the gut sinus by antiperistaltic waves of contraction of the gut muscle coat, which lies outside the sinus (Hanson, 1948*a*). I shall show in a later paper that all serpulid blood-vessels have muscle-fibres in their walls.

1. *Pomatoceros*

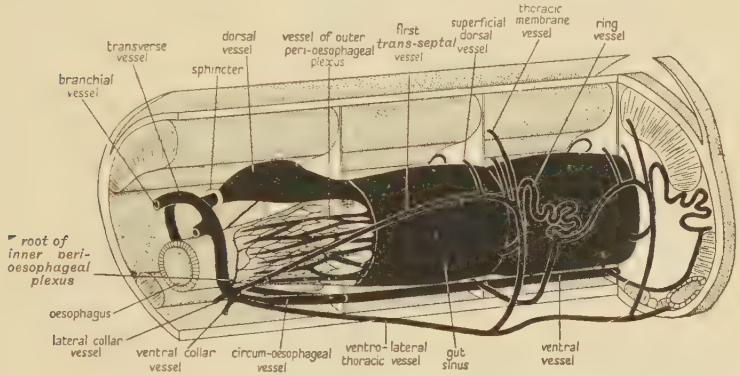
The central blood-system of *Pomatoceros* is diagrammatically represented in Text-figs. 1–4.

The gut sinus and the ventral vessel extend from the posterior end of the penultimate abdominal segment (Text-fig. 1) to the anterior end of the second thoracic segment (Text-fig. 2). In each segment the ventral vessel communicates with the ventro-lateral part of the sinus by a pair of ring vessels, which lie on the anterior face of the posterior septum of the segment (Text-figs. 3 and 4). The thoracic ring vessels are longer than those of the abdomen and are looped;

parts of them are suspended in the coelom by mesenteries attached to the septa. The chloragogen tissue of *Pomatoceros* is situated on the thoracic ring vessels and on the first few abdominal ring vessels. The gut sinus leads anteriorly into a dorsal vessel lying above the oesophagus and terminating in



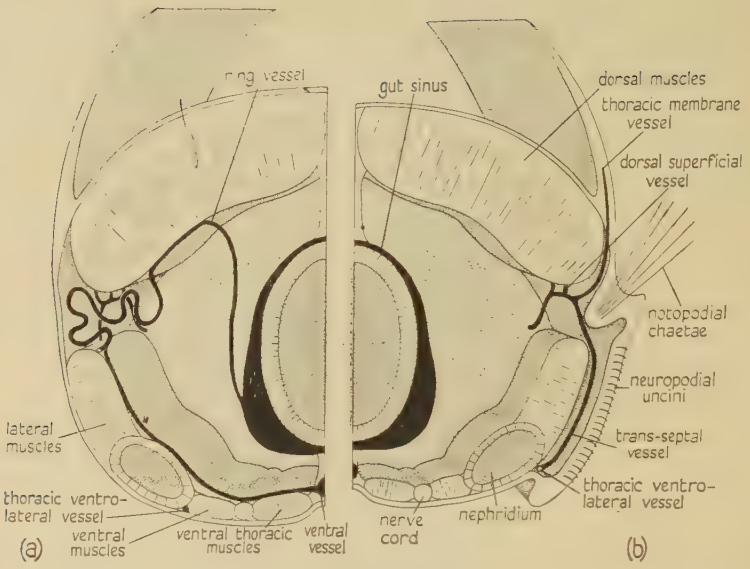
TEXT-FIG. 1. Diagram of blood-system at posterior end of *Pomatoceros triqueter*, in ventral view.



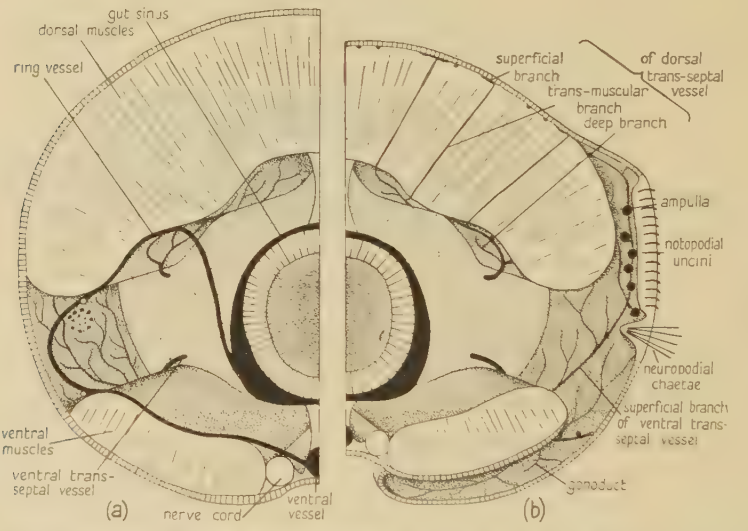
TEXT-FIG. 2. Diagram to show arrangement of vessels in peristomial and second and third thoracic segments of *Pomatoceros triqueter*. Lateral view, with half body-wall removed.

a transverse vessel situated just behind the cerebral ganglia. Each end of the transverse vessel bifurcates into a branchial vessel and a circum-oesophageal vessel. The latter passes downwards through the peristomial cavity, enters the connective tissue lying under the oesophagus (Text-fig. 7), and extends backwards through this tissue to the posterior end of the peristomium, where it joins its fellow from the other side to form the ventral vessel.

The anterior part of the dorsal vessel (Text-fig. 5) is surrounded by a sphincter muscle, and contains a valve which probably ensures that blood returning from the branchial crown does not enter the dorsal vessel, but is directed through the circum-oesophageal vessels to the ventral vessel. A

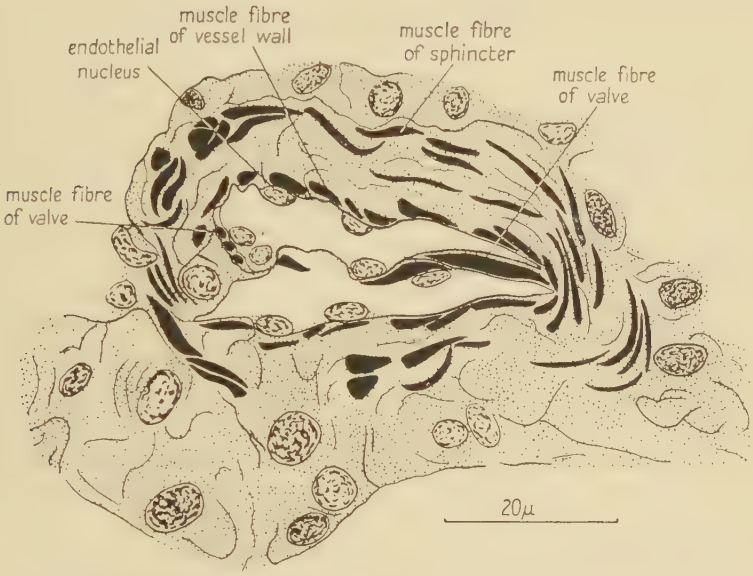


TEXT-FIG. 3. Diagrams to show arrangement of vessels in one thoracic segment of *Pomatoceros triqueter*, viewed from behind, (*a*) whole length, (*b*) half length of segment.



TEXT-FIG. 4. Diagrams to show arrangement of vessels in one abdominal segment of *Pomatoceros triqueter*, viewed from behind, (*a*) whole length, (*b*) half length of segment.

transverse section through the middle of this region of the dorsal vessel shows that the lumen is separated into two channels, dorsal and ventral, by a horizontal septum. Posteriorly, the septum slopes downwards and fuses with the floor of the vessel. Anteriorly, it slopes upwards and ends in a free edge where the dorsal vessel enters the transverse vessel. Clearly this septum can impede the back-flow of blood from the transverse vessel into the dorsal vessel without hindering the flow of blood in the opposite direction. The valve consists of a thin sheet of connective tissue enclosing muscle-fibres and



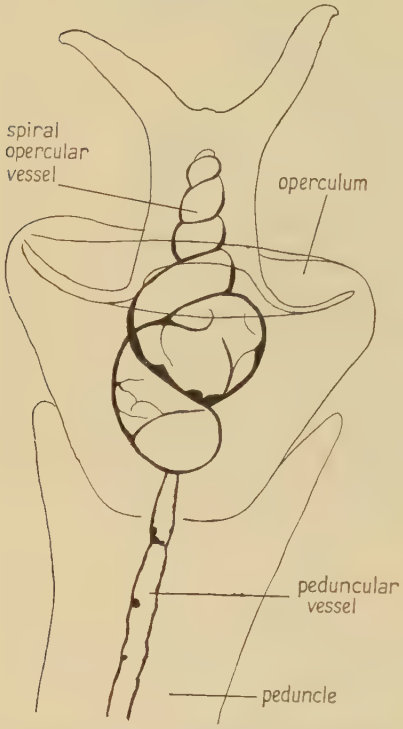
Text-fig. 5. Obliquely transverse section through anterior part of dorsal vessel of *Pomatoceros triqueter*, to show valve and sphincter.

covered on each surface by an endothelium. The muscle-fibres enter the valve antero-laterally and pass obliquely backwards to converge towards the median line. Their contraction will presumably flatten the valve and facilitate the flow of blood out of the dorsal vessel.

The anterior end of the dorsal vessel is not visible in the living animal. The movements of the valve have therefore not been observed, nor has it been possible to discover the circumstances in which the sphincter contracts. However, the following suggestion may be made. By watching animals living in glass tubes, it has been found that movement of blood in the vessels of the crown ceases soon after it has been withdrawn into the tube; but blood continues to move forwards through the gut sinus. It is possible that the sphincter muscle contracts at this time and prevents the flow of blood into the branchial vessels from the dorsal vessel. Its contraction would divert blood through the thoracic ring vessels from the gut sinus to the ventral vessel. Unfortunately the thorax of *Pomatoceros* and the other serpulids which possess the sphincter

is too opaque for this suggestion to be tested by direct observation. The sphincter muscle was noticed by Thomas (1940). The valve has not previously been described in *Pomatoceros*, but was found by Lee (1912) in *Protula intestinum*.

The peripheral blood-system of *Pomatoceros* is diagrammatically represented in Text-figs. 2–4 and 8 and 11. The components of the peripheral blood-system are as follows: the branchial vessels and their branches in the crown; the vessels supplying the collar, lips, and anterior part of the alimentary canal; the peri-oesophageal plexuses; the trans-septal vessels with branches in the body-wall, parapodia, and thoracic membrane.



TEXT-FIG. 6. Benzidine preparation of operculum of *Pomatoceros triqueter*. Vessel distended with gas, and blood adhering to walls. × 35.

The two branchial vessels leave the central blood-system where the circum-oesophageal vessels join the transverse vessel (Text-fig. 2). They enter the crown dorsally, then bend in a ventral direction; they follow the curvature of the base of each half of the crown, supply one vessel to each filament, and end in the vessels of the last filaments. *Pomatoceros* does not possess branchial vesicles, i.e. the vesicular proximal portions of the branchial vessels found in some sabellids. The filament vessels send one branch to each pinnule. The vessel of the most dorsal filament of each side gives off one branch to the adjacent 'palp'. Elsewhere (Hanson, 1949) I have suggested that the 'palp' is a modified pinnule. The peduncle of the operculum receives a branch of the left branchial vessel. In the operculum it ends in a wide, thin-walled, blind-ending vessel, which is coiled in a spiral round a core of connective tissue and reaches nearly to the roof of the operculum (Text-fig. 6). A spiral opercular vessel has not previously been recorded in any serpulid, but the figure published by McIntosh (1926) of a longitudinal section through the operculum of *Mercierella enigmatica* suggests that a spiral vessel also exists in this species. In most serpulids the opercular vessel has branches ending in ampullae.

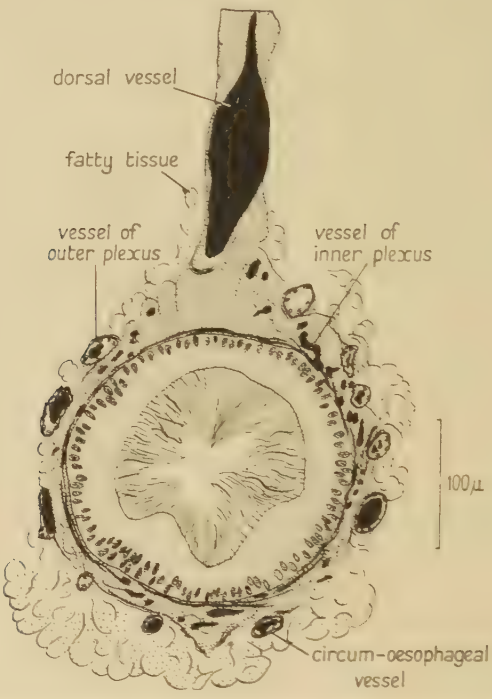
The good blood-supply of the serpulid operculum has naturally suggested to previous workers (Örley, 1884; zur Loye, 1908; McIntosh, 1918, 1926) that it may have a respiratory function, particularly when the crown has been

withdrawn into the tube and the operculum blocks the mouth of the tube. However, observations made on *Pomatoceros* living in glass tubes have shown that soon after the crown has been retracted the movement of blood ceases in all its vessels, including the peduncle vessel. *Sabella* behaves similarly (Fox, 1933). Therefore neither the operculum nor the rest of the crown functions as a respiratory organ when the crown is inside the tube. The movement of blood in the capillaries of the thoracic membrane and body-wall continues, and respiratory exchange is probably carried out between the blood in these vessels and the surrounding water, which is kept moving through the tube by vigorous pumping movements of the abdomen (as in *Sabella*—Nicol, 1930; Fox, 1938) and also by the activity of the ciliary tracts described by Segrove (1938) and Thomas (1940).

The two collar vessels, ventral and lateral, leave each circum-oesophageal vessel near the place where it enters the connective tissue around the oesophagus (Text-figs. 2 and 11). The lateral collar vessel supplies the capillaries on the lateral surface of the peristomium and in the lateral part of the collar. The ventral collar vessel supplies the ventral part of the collar. Soon after leaving the circum-oesophageal vessel it gives off a branch which supplies the connective tissue around the cerebral ganglia, the anterior part of the oesophagus and the buccal cavity, and ends in the capillaries of the dorsal and ventral lips.

A transverse section through the middle of the oesophagus (Text-fig. 7) shows the following layers in the oesophageal wall: the epithelium lining the lumen; a thin layer of connective tissue with a few thin-walled capillaries; a layer of circular muscles; a thicker layer of connective tissue containing numerous thin-walled capillaries; a sheath of connective tissue fibres; a layer of fatty connective tissue in which lie the circum-oesophageal vessels and several thick-walled vessels on either side of the oesophagus. These thick-walled vessels branch and anastomose with each other to form a two-dimensional plexus originating posteriorly from the gut sinus and ending blindly anteriorly (Text-fig. 2). The numerous thin-walled capillaries lying deeper in the wall of the oesophagus form a three-dimensional plexus ending blindly posteriorly and communicating anteriorly with the circum-oesophageal vessels by a small vessel on each side of the body (Text-fig. 2). There is no communication between the inner and outer peri-oesophageal plexuses. The difference in thickness of the walls of the two systems of vessels is very conspicuous. It is probable that blood-pressure is higher in the outer plexus, which is a blind-ending prolongation of the gut sinus, than it is in the inner plexus, which is supplied by one of several branches of each circum-oesophageal vessel. It may be suggested that the inner plexus constitutes the blood-supply of the oesophageal wall, whilst the outer plexus is more important as a place where blood can be received from the sinus whenever its exit from the anterior end of the dorsal vessel is prevented, for example during contraction of the sphincter muscle around the dorsal vessel.

A double peri-oesophageal plexus has not previously been described in serpulids or sabellids. Haswell (1885) noticed the outer plexus of *Pomatoceros* and found that it originated in the gut sinus; but he thought that its vessels enter the dorsal vessel anteriorly. Thomas (1940) noticed smaller inner vessels and larger, more superficial vessels in the oesophageal wall, but assumed that they constitute a single plexus communicating posteriorly with the gut sinus and ending blindly anteriorly.

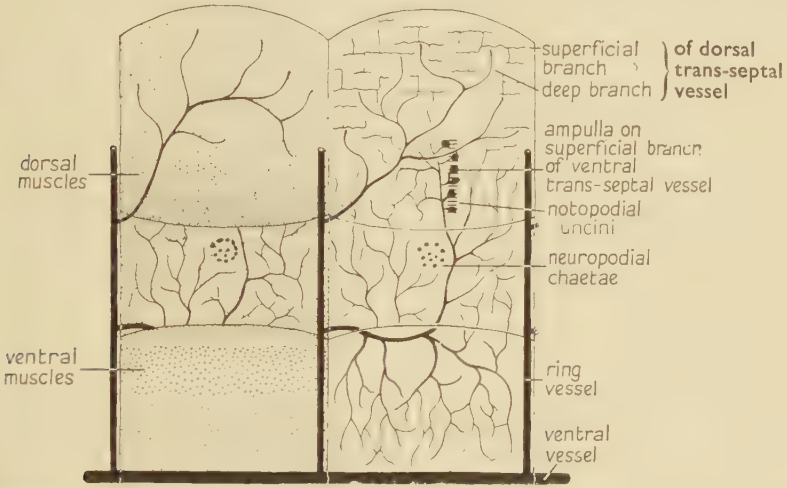


Text-fig. 7. Diagram of transverse section through oesophagus of *Pomatoceros triqueter*.

The blood-supply of the body-wall, parapodia, and thoracic membrane is derived from trans-septal vessels, each of which has its root in a ring vessel of the preceding segment (Text-figs. 2–4 and 8). Each thoracic segment behind the peristomium is supplied by one pair of trans-septal vessels; the first pair are branches of the circum-oesophageal vessels. Each abdominal segment, except the anal segment (Text-fig. 1), is supplied by two pairs of trans-septal vessels, dorsal and ventral. All branches of the abdominal trans-septal vessels end blindly. The main branch of each thoracic trans-septal vessel, on the other hand, ends ventrally in a superficial longitudinal vessel situated in the angle between the ventral surface of the body and the flange at the ventral ends of the uncinal ridges. These two ventro-lateral thoracic vessels (Text-figs. 2, 3, and 11) originate anteriorly in the circum-oesophageal vessels and end in blind-ending branches in the ventro-posterior part of the thoracic membrane.

Branches from these vessels supply the ventral surface of the thorax, including the peristomium.

A thoracic trans-septal vessel leaves the ring vessel at the upper edge of the lateral longitudinal muscle block (Text-fig. 3). It passes through the septum and branches in the next segment. The main branch passes ventrally through the connective tissue around the muscles of the notopodial chaetae. It gives off branches to these muscles, and short, wide, blind-ending capillaries lying under the neuropodial uncini. It ends in the ventro-lateral thoracic vessel.



TEXT-FIG. 8. Diagram to show blood-system of abdominal body-wall of *Pomatoceros triqueter*. Wall cut mid-dorsally and spread flat. Right half of two segments seen from inside. Muscles of one segment depicted as transparent.

Soon after passing through the septum it gives off one large branch to the thoracic membrane and two or three smaller branches which end in capillaries situated just under the basement membrane of the epidermis on the dorsal surface (Text-figs. 2 and 3). Thomas (1940) has stated that the first thoracic membrane vessel is a branch of the circum-oesophageal vessel. I have found that it is a branch of the first trans-septal vessel, which originates in the circum-oesophageal vessel.

An abdominal ventral trans-septal vessel leaves the ring vessel at the lateral edge of the ventral longitudinal muscle block (Text-figs. 4 and 8), passes through the septum, and branches in the next segment into superficial capillaries in the ventral, lateral, and latero-dorsal regions of the body-wall. It also gives off short, wide, blind-ending capillaries situated under the notopodial uncini and projecting into the cavity of the segmental organ. (The segmental organ is a lateral pouch of the coelom extending dorsally into the parapodium and ventrally under the body-wall as a gonoduct, which opens to the exterior near the mid-ventral line.)

An abdominal dorsal trans-septal vessel leaves the ring vessel at the lateral

edge of the dorsal longitudinal muscle block (Text-figs. 4 and 8). There are sometimes two, rarely three, dorsal trans-septal vessels originating in one ring vessel. After passing into the next segment the vessel branches over the coelomic surface of the dorsal muscle block. Each of these branches bends at right angles and, without branching, penetrates through the muscle block by way of the connective tissue separating the muscle compartments, and then emerges under the epidermis of the dorsal surface of the body. Here it gives off a few blind-ending branches which do not anastomose with the capillaries of the ventral trans-septal vessel. These superficial capillaries of the dorsal trans-septal vessels are easily recognized (Text-fig. 8). They lie either parallel to or at right angles to the longitudinal muscle-fibres, and the longitudinal capillaries become sinuous when the muscles contract. The ventral trans-septal vessels, on the other hand, branch at acute angles.

A few capillaries lie just underneath the epithelium of the excretory sacs of the thoracic nephridia, but I have been unable to find their source.

Lateral vessels, such as are found in *Sabella*, are absent in *Pomatoceros* and all the other serpulids I have examined. It is probable that Meyer (1888), Lee (1912), and Thomas (1940) mistook trans-septal vessels for lateral vessels.

2. *Other serpulids*

The central blood-system of all the serpulids examined is like that of *Pomatoceros*, except for differences in the valve and muscular sphincter of the dorsal vessel. *Hydroides*, *Serpula vermicularis*, and *Vermiliopsis* possess both valve and sphincter. *Protula intestinum* possesses the valve but lacks the sphincter. *Spirorbis militaris* and *Salmacina* lack both valve and sphincter. Suitable serial sections of the dorsal vessels of other serpulids have not been prepared.

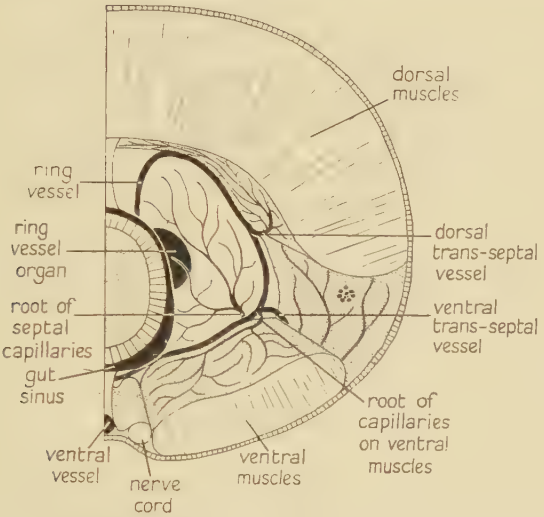
Whereas the central blood-system appears to be uniform throughout the family, variations have been found in the peripheral blood-system. Some of these variations are attributable to differences in body size, and others are at present inexplicable from a functional point of view.

Pomatoceros is unusual in possessing a spiral opercular blood-vessel. In other genera the vessel bears branches ending in ampullae. These vessels have been described in *Hydroides norvegica* by Okada (1932–3), *H. lunulifera* by Claparède (1868–70), *H. uncinata* and *Serpula vermicularis* by Örley (1884), *Apomatus similis* by de St. Joseph (1894), and *A. ampulliferus* by Zeleny (1905). The opercular vessel of *Serpula lo-biancoi* is like that of *S. vermicularis*. In *Vermiliopsis infundibulum* the vessel branches just under the chitinoid cap of the operculum into a number of short vessels ending in ampullae.

Serpula vermicularis possesses a double peri-oesophageal plexus like that of *Pomatoceros*, but I have been unable to discover if the inner plexus is connected with the circum-oesophageal vessels. The outer plexus is absent in *Hydroides*, *Vermiliopsis*, and *Protula intestinum*. I have been unable to trace the origin of the small vessels found in the oesophageal wall of these serpulids. *Salmacina* and *Spirorbis militaris* lack peri-oesophageal plexuses.

The blood-supply to the body-wall in *Salmacina* and *Spirorbis* is greatly reduced, presumably because of the small size of these serpulids. *Salmacina* is 2–3 mm. long, *Spirorbis* even smaller. There is a single pair of unbranched blind-ending trans-septal vessels in each segment, extending from the ring vessels of one segment to the parapodia of the next posterior segment. Ventro-lateral thoracic vessels are absent.

Protula intestinum, 8–12 cm. long, is considerably larger than other serpulids. Some special features of the abdominal peripheral blood-system are



TEXT-FIG. 9. Diagram to show vessels in one half of an abdominal segment of *Protula intestinum*, seen from behind. Anterior septum depicted as transparent.

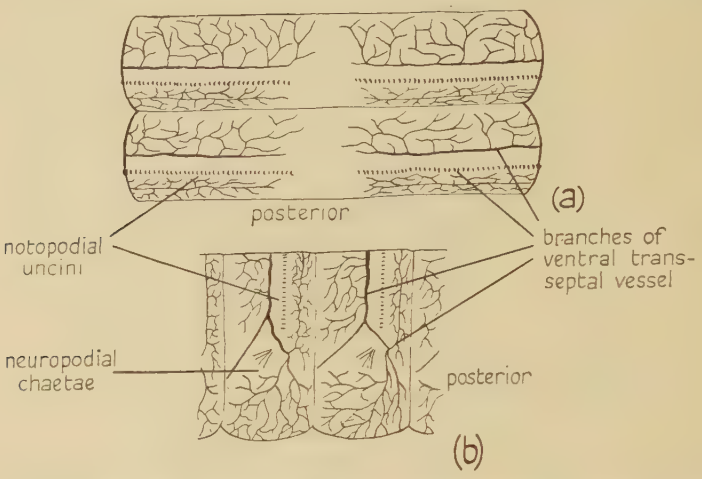
probably attributable to its large size; they are not found in *P. tubularia* which is much smaller. The abdominal intersegmental septa, and the coelomic surface of the abdominal ventral body-wall have a rich blood-supply derived from two vessels not found in other serpulids (Text-fig. 9). Just before the ventral trans-septal vessel passes through the septum it gives off a branch which is also trans-septal, and supplies the coelomic surface of the ventral muscle block. The capillaries of the septum lie on its anterior face and lead into a small vessel which opens into the ring vessel just proximal (more towards the mid-ventral line) to the place of origin of the ventral trans-septal vessel.

Protula intestinum and *Apomatus ampulliferus* possess organs of unknown function on the abdominal ring vessels close to the gut sinus. They resemble the 'blood glands' of *Pheretima* (Stephenson, 1924). They have not been found in other serpulids. They will be described in a later paper.

Dorsal abdominal trans-septal vessels are absent not only in *Salmacina* and *Spirorbis*, but also in the larger species *Serpula lo-biancoi* (Text-fig. 10) and *Vermiliopsis*. In these two species the vessels which supply the ventral and

lateral surfaces of the abdominal body-wall also supply the dorsal surface. *Serpula vermicularis*, unlike *S. lo-biancoi*, possesses dorsal trans-septal vessels. It also differs from *S. lo-biancoi* and from all other serpulids in that the superficial branches of the dorsal trans-septal vessels are connected with each other, on each side of the body, by a longitudinal vessel, which also receives the main dorsal branches of the ventral trans-septal vessels.

On the ventral surface of the thorax of all serpulids except *Salmacina* and *Spirorbis* there are numerous blood-vessels situated just under the epidermis.



Text-fig. 10. Diagrams of abdominal superficial blood-system of *Serpula lo-biancoi*, (*a*) in dorsal, (*b*) in lateral view.

and visible in living animals. This system of vessels is useful in the identification of specimens, because the pattern varies from species to species, and is invariable in its main features within any one species. Diagrams of these vessels are given in Text-figs. 11–18.

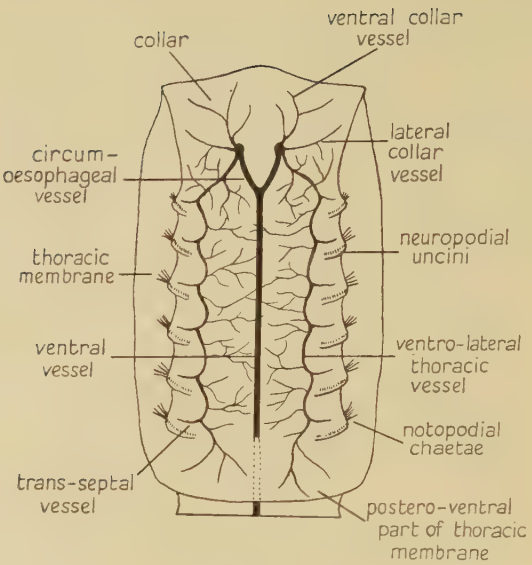
In *Pomatoceros triqueter* (Text-fig. 11) three longitudinal vessels are visible, the ventral vessel and the two ventro-lateral thoracic vessels. These three vessels are not directly connected with each other. The ventro-lateral vessels originate in the circum-oesophageal vessels and end blindly in the ventro-posterior part of the thoracic membrane. Two pairs of collar vessels, ventral and lateral, leave the circum-oesophageal vessels. In *Hydroides norvegica* (Text-fig. 12), *Serpula vermicularis*, and *S. lo-biancoi* (Text-fig. 13) each ventro-lateral vessel is directly connected with the ventral vessel by a superficial transverse vessel at the posterior end of each segment. The ventro-lateral vessels begin anteriorly in the first transverse vessel and end posteriorly in the last transverse vessel. In *H. norvegica* and *S. vermicularis* the ventro-posterior part of the thoracic membrane is supplied by two branches of the last transverse vessel. *S. lo-biancoi*, like *Vermiliopsis infundibulum* (Text-fig. 14), lacks the ventral part of the thoracic membrane. In *V. infundibulum* the

ventro-lateral vessels are situated farther away from the uncinal ridges than in other serpulids. The main collar vessel of *H. norvegica* is an anterior prolongation of the ventral vessel. *S. vermicularis* resembles *H. norvegica* except for the absence of this conspicuous collar vessel. In *S. lo-biancoi* a similar vessel supplies the surface of the peristomial segment which, in this species, is longer than usual.

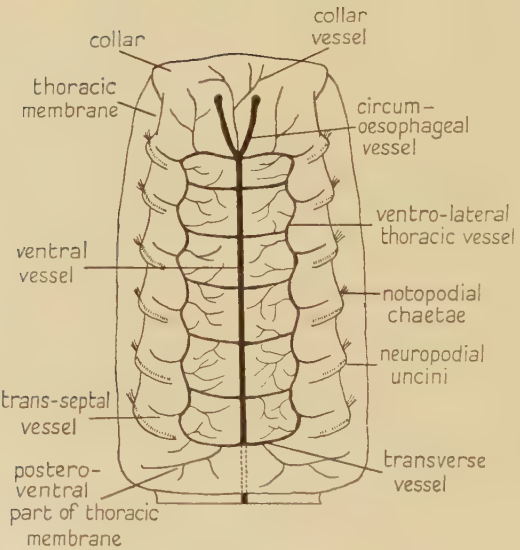
Protula (Text-figs. 15 and 16) and *Apomatus* (Text-figs. 17 and 18) differ from other serpulids in the absence of uncinal ridges. *P. intestinum* (Text-fig. 15) is conspicuously different in that it lacks ventro-lateral thoracic vessels; the trans-septal vessels extend across the ventral surface of the thorax and enter the ventral vessel. The ventro-posterior part of the thoracic membrane is supplied by two branches of the ventral vessel. *A. ampulliferus* (Text-fig. 18) is unusual in that the first two pairs of trans-septal vessels join the circum-oesophageal vessels instead of the ventro-lateral vessels. *P. intestinum* is in many ways different from *P. tubularia*, and *P. tubularia* in many ways closely resembles *Apomatus*. Elsewhere (Hanson, 1948*b*) I have discussed the relationship of the two genera and suggested that they need to be revised.

Faulkner (1930) has published an account of the blood-system of *Filograna implexa* and *Salmacina dysteri* in which she had described a system of sinuses which only in the anterior part of the body become restricted to form vessels. The sinus around the alimentary canal spreads into sinuses in the septa and mesenteries. These extend into sub-epidermal sinuses. The ventral mesenteric sinus takes the place of a ventral vessel. The ring vessels are wide parts of the septal sinuses, and they connect the sinuses in the dorsal and ventral mesenteries. The ring vessels sometimes have backward-pointing caeca. Anteriorly there are distinct dorsal, circum-oesophageal, and branchial vessels arranged in the usual manner. I have been unable to find mesenteric, septal, or sub-epidermal sinuses in *Salmacina incrustans*. In living specimens one can see a well-defined ventral vessel connected by discrete ring vessels with the gut sinus; each ring vessel gives off an unbranched trans-septal vessel which ends blindly near the parapodium of the next posterior segment. These observations have been confirmed in benzidine preparations and serial sections.

In benzidine preparations, Faulkner observed superficial blue-coloured patches, and in untreated animals the epidermal cells appeared to be outlined in pale green, as though blood containing chlorocruorin were present between the basal parts of the cells. From these observations she concluded that extensive sub-epidermal blood sinuses are present. I have confirmed her observations, but prefer an alternative explanation. The cell outlines are pale green in colour not only in surface view, but also in profile view; and in the latter case the outer margin of the cell also has the pale green colour. The colour therefore seems to be a property of the cell surface and is not necessarily due to the presence of a sub-epidermal blood sinus. The benzidine reaction is not specific for blood pigments, but is a reaction for all peroxidases (Prenant, 1924).

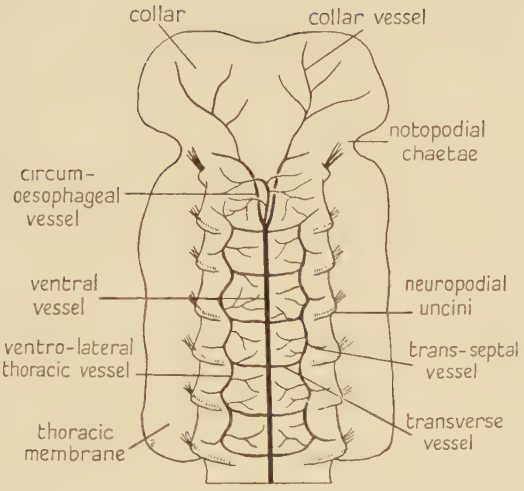


TEXT-FIG. 11

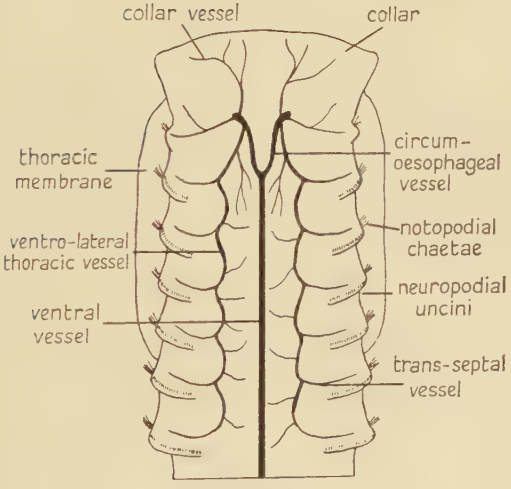


TEXT-FIG. 12

TEXT-FIGS. 11–12. Diagrams of ventral thoracic superficial blood-systems of: 11, *Pomatoceros triqueter*; 12, *Hydroides norvegica*.



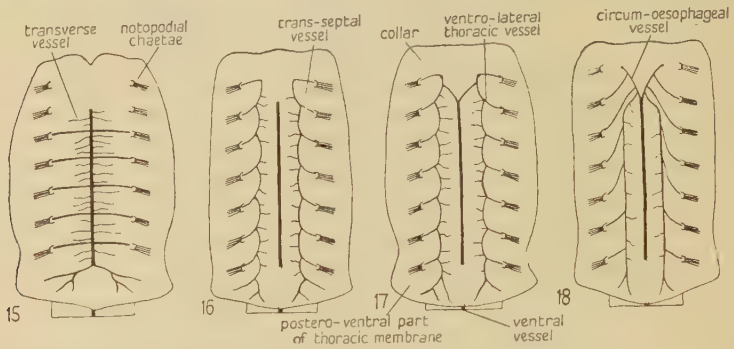
TEXT-FIG. 13



TEXT-FIG. 14

TEXT-FIGS. 13–14. Diagrams of ventral thoracic superficial blood-systems of: 13, *Serpula lo-biancoi*; 14, *Vermiliopsis infundibulum*.

In benzidine preparations the vessels often have irregular outlines, and the blue-black granules are not uniformly distributed; uncoloured patches are often found. Faulkner has concluded from this appearance that the blood is not confined in well-defined vessels, but is in sinuses; for example, there is a ventral mesenteric sinus instead of a ventral vessel. However, the benzidine



TEXT-FIGS. 15–18

Diagrams of ventral thoracic superficial blood-systems of: 15, *Protula intestinum*; 16, *P. tubularia*; 17, *Apomatus similis*; 18, *A. ampulliferus*.

reaction is violent, and numerous bubbles of oxygen are liberated (see Text-fig. 6). The irregular appearance of vessels after the reaction is an artifact, although the general anatomy of the blood-system is usefully demonstrated by this method. Careful examination of living *Salmacina incrustans* leaves no doubt that its vessels have well-defined walls and are not sinuses.

I acknowledge with gratitude the help of Professor H. Munro Fox who suggested, encouraged, and usefully criticized the research reported in this paper. I am also indebted to Dr. A. Stock for many discussions of problems and results, to the British Association for the Advancement of Science for the use of its Table at the Zoological Station of Naples, to the staff of the Station for the facilities they provided, and to the University of London for a grant which enabled me to go to Naples.

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A Study of the Testis Tubules, Interstitial Tissue, and Sex Characters (Thumb-pads and Wolffian Ducts) of Normal and Hypophysectomized Frogs (*Rana esculenta*)

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Summary

1. After hypophysectomy in October the testes and some sex characters (Wolffian ducts and thumb-pads) of *Rana esculenta* were investigated during 9 subsequent months.

2. The so-called prespermatogenesis (Champy, 1913) is completed normally after hypophysectomy, but the primary spermatogonia lose their capacity for division. Therefore 8 months after the operation the testis tubules contain only primary spermatogonia.

3. The spermatogonia of the testes of control frogs, kept under laboratory conditions from October onwards, show a precocious activity during the winter, but in July new spermatozoa have not yet been formed.

4. In both control and experimental frogs the spermatozoa, formed during late summer and autumn, almost entirely disappear in the spring, even in the absence of copulations.

5. During the winter and spring months the interstitial testis cells of the control frogs undergo a reduction in both number and function, and this coincides with the increased activity in the testis tubules which occurs under laboratory conditions.

6. Conversely the decreased spermatogenetic activity, caused by hypophysectomy, coincides with an increase in the number of interstitial cells. The cytoplasm of these cells, however, is greatly reduced, although the nuclei maintain their typical form and size till at least 9 months following the operation.

7. Thumb-pads and Wolffian ducts are strongly affected by hypophysectomy.

8. The cytological changes brought about in the interstitial cells of *R. esculenta* after hypophysectomy do not quite coincide with changes in the development of the thumb-pads and Wolffian ducts. Therefore no new evidence for the endocrine function of these cells can be given.

Contents

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Introduction

THE present paper is one of a series in which the endocrine function of the interstitial tissue of the testis is being examined. It also forms part of a second series, in which it is intended to deal with the regulation of spermatogenesis, and in which the phenomenon of spermiation in *Rana* and *Salamandra* (van Oordt, Creutzberg, and Spronk, 1949) has already been described.

In the papers on the interstitial tissue of mammals (Sluiter, 1945) and of birds (Sluiter and van Oordt, 1947, 1949) conclusions were reached regarding its endocrine function and regarding the cells in which the hormone is formed. A comparison was made between the cytology of normal interstitial cells and those from animals treated with gonadotropins, the state of the sex accessories serving as an indicator for the hormone secretion.

In the present paper the reverse experiment, i.e. hypophysectomy, has been used to obtain further information.

So far a detailed cytological description of the interstitial cells of the frog's testis, following hypophysectomy, has not been published. However, the changes in the gonads of hypophysectomized frogs have already been investigated by Gallien (1940) in *Rana temporaria*, but as the sexual cycles in the testes of both *Rana*-species are very different (Champy, 1913) a careful study of the testes of *R. esculenta* after hypophysectomy seems important.

Moreover, the well-known fact (Harms, 1926) must be taken into account that confinement during the winter months has a great influence on frogs. When they are protected against extremely low outdoor temperatures and fed regularly, frogs acquire a sexual cycle which differs completely from that of frogs hibernating in nature. Consequently, in the present paper we shall compare three different groups of frogs:

(1) animals recently caught in the wild ('normal animals'),

(2) animals kept for some time in the laboratory at room temperature and fed regularly ('controls'), and

(3) hypophysectomized animals kept in the laboratory under the same conditions ('experimentals').

Material and Method

On 11 and 13 October 1948, some days after they were caught, the experimental frogs were hypophysectomized, using the method of Mighorst (Sluiter, Mighorst, and van Oordt, 1949). They were then kept, together with the controls, in a large vivarium at room temperature and fed with mealworms, which were taken actively. The mortality was rather high only during the first few days following the operation, and several experimentals lived till July of the next year.

Both experimentals and controls were killed regularly during the period from October till July, the intervals varying from 2 or 3 weeks to 2 months.

Of each animal one testis, both thumb-pads, and both Wolffian ducts were fixed in Bouin's solution, sectioned (7 or 10μ), and stained with haemalum-eosin. The other testis was used for detailed cytological investigations and was fixed in Kolster's fluid, sectioned at 3μ, and stained with Altmann's acid-fuchsin, combined with brilliant-cresyl-blue.

Results

Testes

Testis Tubules

Spermatogenesis. According to Champy (1913) a so-called prespermatogenesis occurs in *R. temporaria* as well as in *R. esculenta*. It was described as the process whereby the primary spermatogonia do not develop further than into primary spermatocytes, which soon degenerate. As no spermatozoa are formed during this process we think it better to call it prespermatogenetic activity.

In *R. temporaria* it was found (Champy, 1913) that prespermatogenetic activity occurs only during May and June. The formation of spermatozoa takes place in July, August, and September. During autumn and winter very many bundles of spermatozoa are present in the testis tubules, but no spermatogenetic activity occurs. On the other hand, Champy (1913) found that in *R. esculenta* the prespermatogenetic activity continues all the year round, with the exception of the period from July till October, when spermatozoa are formed. Consequently, during the whole year the testis tubules of *R. esculenta* contain cell nests which are derived from spermatogonia by subsequent simultaneous divisions.

In order to determine the prespermatogenetic activity quantitatively, Champy (1913) counted the number of degenerating spermatocytes present. This complicated method was not used by the present authors. Instead, the testis tubules were divided into six different stages after estimations had been made of the number of spermatogenetic cells present in each cross-section. These tubules have been classified as follows (cf. Text-fig. 1):

Stage-number 0: testis tubule with a few primary spermatogonia present.

" " 1: testis tubule with primary spermatogonia and one small cell nest.

" " 2: testis tubule with primary spermatogonia and several small cell nests.

" " 3: testis tubule with primary spermatogonia, several small and at least one large cell nest.

" " 4: testis tubule with a closed row of cell nests at its periphery.

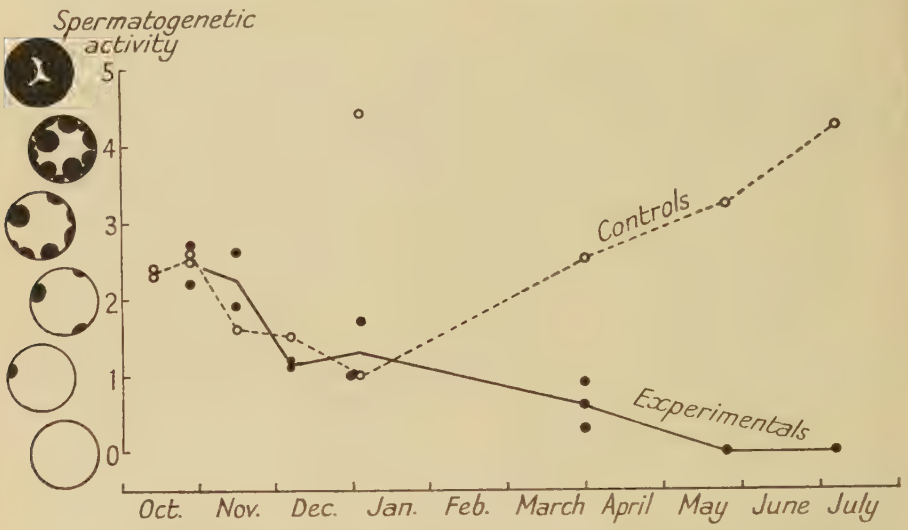
" " 5: testis tubule almost totally filled with cell nests.

In order to determine the spermatogenetic activity of a testis, the various stages present in twenty cross-sectioned testis tubules were estimated. Then

the average stage-number of these twenty tubules was taken as representing the spermatogenetic activity of the gonad as a whole. As a precaution several testis sections were used for each estimation, as in every testis section between three and six different tubule stages are generally to be found.

Text-fig. 1 shows the spermatogenetic activity of controls and experimentals.

Controls. During the period October–January a gradual decrease in prespermatogenetic activity takes place, as in normal animals (cf. Champy, 1913,



Text-fig. 1. *Rana esculenta.* Graph showing spermatogenetic activity from October to July in hypophysectomized frogs ('experimentals') and in frogs living under laboratory conditions ('controls'). For explanation of the circles cf. text, p. 133.

graph I, p. 46). Thus confinement, a relative high temperature, and regular feeding have no influence on the prespermatogenetic activity during this period.

From January onwards the reaction of the controls is not so uniform. At the beginning of January the spermatogenetic activity of one of the controls was very strong, but that of another, representing the normal state, was weak.

In control specimens, killed at the end of March, the end of May, and the beginning of July, the spermatogenetic activity was much stronger than normal. Apparently the laboratory conditions cause a precocious onset of spermatogenesis. In the laboratory spermatogenesis begins as early as February, but in the wild it does not do so before the beginning of July. The process itself is, however, much delayed, because in July spermatozoa were not yet formed in the testis tubules of control frogs.

Experimentals. In the period from October to January the spermatogenetic activity of the experimentals shows the same decrease as that of the controls. Three months after hypophysectomy, however, this decrease still continues so that by May stage 0 is reached.

Therefore we may conclude that the increasing activity which was found in the controls from January onwards is at least partly due to the influence of the pituitary. As the spermatogenetic activity of the controls and experimentals is almost identical from October till January, it is probable that during this period the pituitary has no gonadotropic function.

Spermatozoa. Under natural conditions the testis tubules of *R. esculenta*, like those of other *Rana*-species, contain large quantities of spermatozoa during the winter months. These spermatozoa have been produced in summer and early autumn. In the control specimens, however, the number of spermatozoa varies widely. Whether this variability was due to the laboratory conditions or not could not be ascertained.

In spite of the fact that copulation did not take place, the number of spermatozoa present in the testes of control frogs decreased considerably in May.

As far as could be determined, the quantity of spermatozoa until May was much larger in the experimentals than in the controls. In this month a marked decrease in the number of spermatozoa also occurred in the testes of the experimentals. This suggests that the decrease is not caused by a hypophyseal influence, but may be due to the limited duration of life of the spermatozoa stored in the testis tubules.

Interstitial Tissue

According to Champy (1913) the periodic changes in the interstitial tissue are different in *R. temporaria* and *R. esculenta*. Unlike *R. temporaria*, the interstitial tissue of *R. esculenta* is well developed during the greater part of the year. Only from July till the beginning of October, the spermatogenetic period, is it poorly developed.

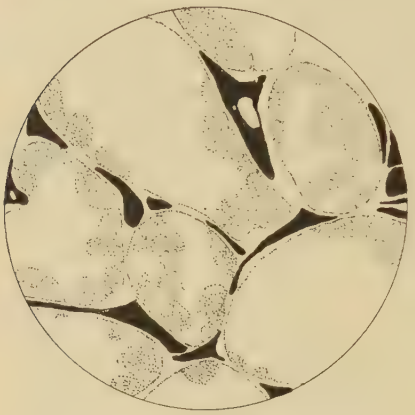
Several times already we have pointed out (Sluiter, 1945; Sluiter and van Oordt, 1947, 1949) that in studying the endocrine function of the testis it is not sufficient to pay attention only to the volume of the total intertubular tissue. It is generally known that in most of the vertebrates interstitial cells can be distinguished, which by their structure and contents suggest a secretory or a storage function. To judge the activity of the intertubular tissue at a particular moment it is therefore necessary to pay attention to the number and to the physiological state of these cells.

During the long period in which the intertubular tissue of normal specimens of *R. esculenta* is well developed it is, according to Champy (1913), composed mainly of large cells, containing lipoid droplets and mitochondria. As spermatogenesis increases during July, the lipoid progressively disappears from the interstitial cells and moves into the testis tubules. At the same time a regression of the interstitial cells occurs, with the resulting formation of connective tissue-like cells. At the end of the spermatogenetic period these cells are again transformed into the active cells, described above.

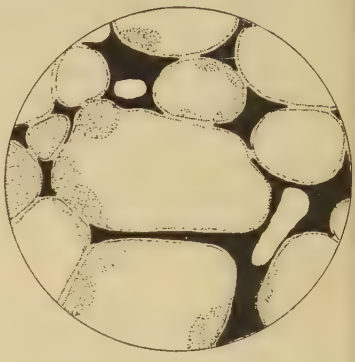
The results of our quantitative investigations into the interstitial cells with regard to their total surface in sections may be seen in Text-figs. 2–5 and 10–

12; the cytological details of these cells are shown in Text-figs. 6–9 and 13–15.

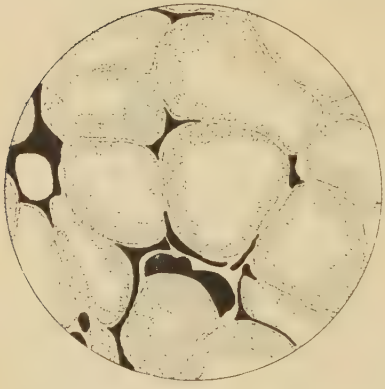
Control-specimens (Text-figs. 2–9). In Text-figs. 2 and 3, showing testis sections of control frogs killed during the autumn and winter months, the



TEXT-FIG. 2



TEXT-FIG. 3



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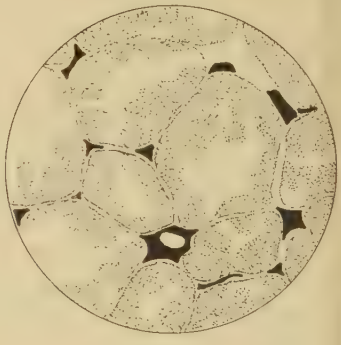


TEXT-FIG. 5

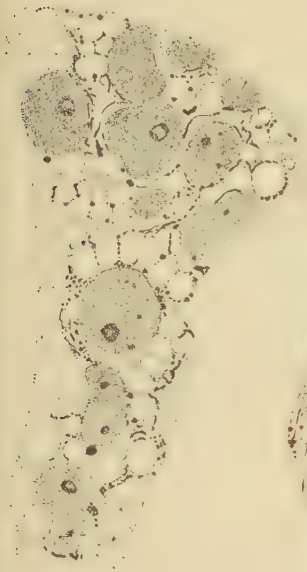
TEXT-FIGS. 2–5. *Rana esculenta.* Testis sections of control frogs, autopsied respectively 12 Oct. 1948, 17 Nov. 1948, 4 March 1949, and 7 July 1949. The diameter of each circle is proportional to the average lengths of the long and short axis of both testes. Black: total surface of the interstitial cells; dotted: spermatogenetic cell nests. Primary spermatogonia and spermatozoa are not drawn. Champy-fixation; Altmann-staining. (All figs. × 120.)

interstitial cells cover a relatively large area of the testis section. In this period they possess a large round nucleus and a cell body filled with many lipoid droplets and mitochondria. The size and contents of these cells vary during this period between the limits drawn in Text-figs. 6 and 7, respectively. This description is in agreement with that of Champy (1913) for normal green frogs from October to July.

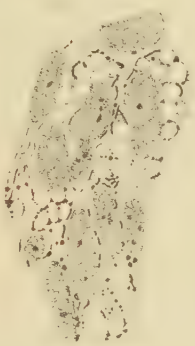
At the end of March, however, the total section area occupied by the interstitial cells of the control frogs is somewhat smaller (Text-fig. 4). This is due

to the fact that most of the interstitial cells have become smaller and more elongated (Text-fig. 8), but they still contain some large lipoid droplets and scattered mitochondria.

In the period from the end of March until July the total section area of the interstitial cells decreases gradually (Text-fig. 5) and the majority of them now take the form of an intertubular connective tissue cell (Text-fig. 9). As stated above, this same regression takes place in the interstitial cells of normal



TEXT-FIG. 6



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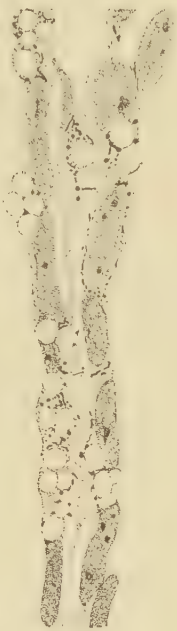
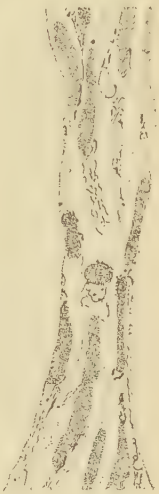


TEXT-FIG. 8



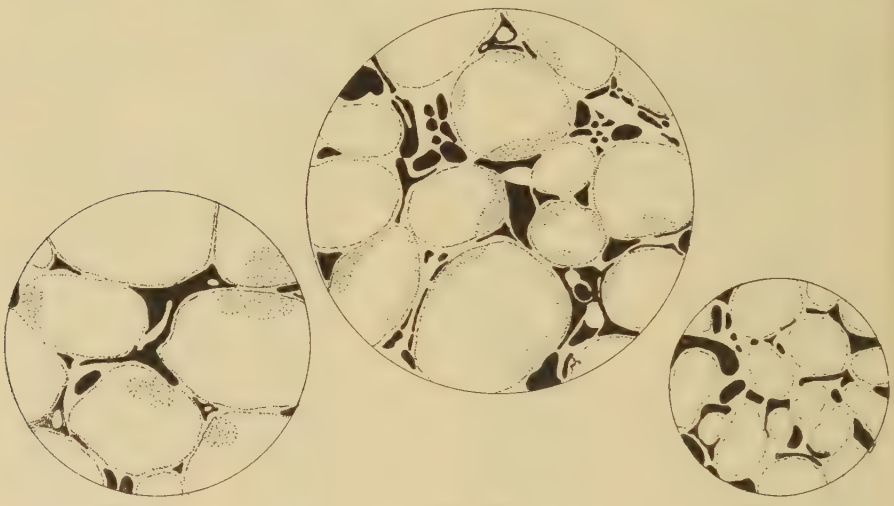
TEXT-FIG. 9

TEXT-FIGS. 6–9. *Rana esculenta*. Interstitial cells of control frogs, autopsied respectively 12 Oct. 1948, 16 Nov. 1948, 29 March 1949, and 29 May 1949. The vacuoles are empty because their contents have dissolved during the preparation of the sections; mitochondria black. Champy-fixation; Altmann-staining. (× 1,800.)

animals, but in these it does not begin until July. Therefore we can conclude that under laboratory conditions the regression of the interstitial cells occurs precociously, and that in laboratory animals it is also accompanied by an increase of the spermatogenetic activity.

Experimentals (Text-figs. 10–15). During the autumn and winter months the total section area of the interstitial cells in the testes of control and experimental specimens is about the same (Text-fig. 10). In spring, however, the section area increases slightly (Text-fig. 11), contrary to what has been described in the controls. In July, about 9 months after hypophysectomy, many interstitial cells are still identifiable as such (Text-fig. 12), but the large cell-groups have divided into small ones (Text-figs. 11 and 12).

In cytological preparations it is obvious as early as 2 weeks after the operation that a varying number of interstitial cells have decreased in size. This decrease affects the cell size, but not the size of the nuclei. Consequently the nuclei lie closer together, the mitochondria are concentrated, and there is no room for lipoid droplets. Moreover, the number of nuclei has markedly increased. Five months after hypophysectomy the condition of Text-fig. 14 is reached. Nine months after the operation the number of cells may have decreased, but the cytological structure is still the same (Text-fig. 15). The



TEXT-FIG. 10 TEXT-FIG. 11 TEXT-FIG. 12

TEXT-FIGS. 10–12. *Rana esculenta*. Testis sections of experimental frogs, hypophysectomized 11–13 Oct. 1948 and autopsied respectively on 7 Dec. 1948, 29 March 1949, and 7 July 1949. For further details cf. legend of figs. 2–5. (×120.)

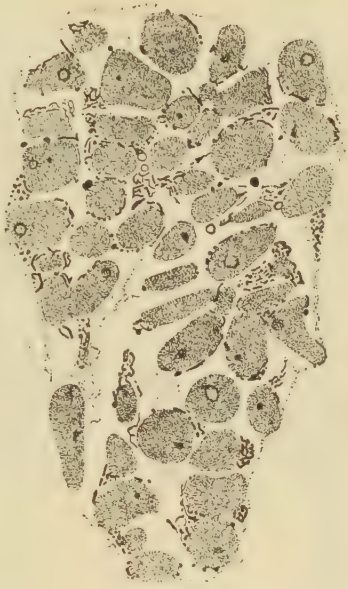
cells are intact, but there is very little physiological activity. Obviously the regression stops at this stage.

As regards some of the experimental animals, autopsied during the first 5 months after the operation, the cytology of the interstitial cells differs markedly from that described above. Excessive numbers of vacuoles develop, while most mitochondria disappear (Text-fig. 13). Occasionally there are so many vacuoles that the nucleus is indented, suggesting a fatty degeneration of the cell.

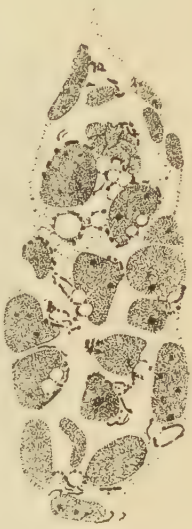
It can be concluded that the regression of the interstitial cells in the testes of hypophysectomized frogs is distinctly different from that of normal and control animals. The cytology of the interstitial cells of the experimentals suggests that possibly the secreting and certainly the storage capacity of the cells decreases. But it is important to notice that in hypophysectomized frogs the number of interstitial testis cells increases strongly during their functional regression. This takes place at a time when the spermatogenetic activity is decreasing.

At the end of the autumn the normal animals also show an increase in the number of interstitial cells and a decrease of spermatogenetic activity, but, unlike the experimentals, the function of these cells is resumed at the same time.

TEXT-FIG. 13



TEXT-FIG. 14



TEXT-FIG. 15

TEXT-FIGS. 13–15. *Rana esculenta.* Interstitial cells of experimental frogs, hypophysectomized on 11–13 Oct. 1948 and autopsied respectively on 7 Dec. 1948, 29 March 1949, and 7 July 1949. For further details cf. legend of figs. 6–9. (×1,800.)

Secondary and Accessory Sex Characters

Experimentally induced changes in the sex characters, viz. in the thumb-pads and Wolffian ducts, may be of special interest, as they can be used as indicators of a changed hormone-production in the testis.

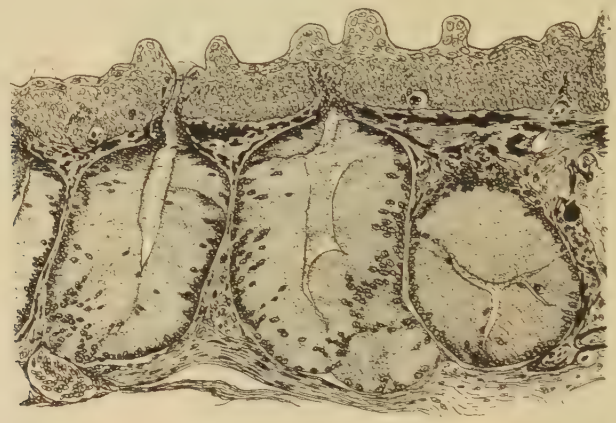
Thumb-pads

As is generally known, the histology of the frog's thumb-pads shows a distinct yearly cycle. After maximum development in the copulation period a marked regression occurs, but during the summer a new development starts, which gradually leads to the maximum condition.

Controls. In October and November a thick corneous cuticle with rather large papillae covers the epidermis, beneath which large glands with high secretory cells are visible (Text-fig. 16). Further development is slow, so that during the copulation period the thumb-pads of the controls do not reach their maximum development, this being probably due to the laboratory conditions.

As a regression of the thumb-pads begins after castration (Harms, 1926) it seems possible that the development of these organs is regulated by the inter-

stitial cells. As a matter of fact, in autumn the quick development of the thumb-pads coincides with the presence of interstitial cells, the cytology of which suggests an active secretory function. However, in our control frogs the thumb-pads may maintain their full development till July, whereas the activity of the interstitial cells disappears in spring. Therefore it cannot be demonstrated from the controls alone that the interstitial cells regulate the development of the thumb-pads.



TEXT-FIG. 16. *Rana esculenta*. Section through a thumb-pad of a control frog fixed on 26 Oct. 1948. Bouin-fixation and haemalum-eosin staining. (× 100.)

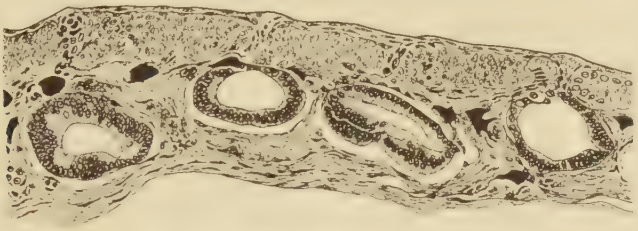
Experimentals. According to Gallien (1940) in *R. temporaria* a regression of the thumb-pads begins very soon after hypophysectomy. Two weeks after the operation, in October 1948, the cuticle of the thumb-pads of our hypophysectomized green frogs was already thin, too; its papillae were lacking and the epidermal glands were small, with low cells and a large lumen (Text-fig. 17). This condition was maintained till July 1949.

Now it might be possible that the regression of the thumb-pads is caused by a decreased function of the interstitial cells, which no longer secrete their androgen after the loss of the gonadotropic potency of the pituitary. However, we have seen (p. 138) that in the experimental frogs, 2 weeks after hypophysectomy, the cytology of the interstitial cells suggests only a small decrease in secretory activity, which is not sufficient to explain the quick and active regression of the thumb-pads. It is therefore impossible to decide with certainty whether this regression is caused by the decreased secretion of androgens in the interstitial cells or not.

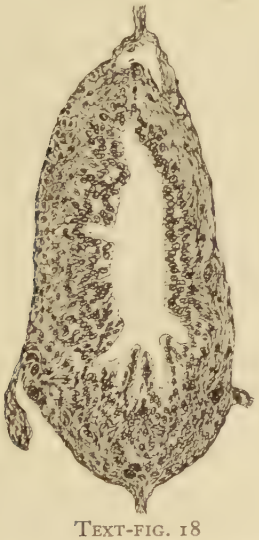
Wolffian Ducts

In the male green frog the Wolffian ducts do not possess seminal vesicles as they do in the grass frog. In the male *R. esculenta* the Wolffian ducts have larger diameters than in the female. Moreover, their interior epithelium shows several folds, which may act as sperm-reservoirs.

In the normal *R. esculenta* periodical changes in the Wolffian ducts similar to those in the seminal vesicle of *R. temporaria* (Harms, 1926) are not known. In control specimens from October to July the epithelium of the Wolffian ducts was always high and folded (Text-fig. 18). Periodical changes could not be demonstrated.



TEXT-FIG. 17. *Rana esculenta*. Section through a thumb-pad of an experimental frog, fixed on 26 Oct. 1948, 14 days after hypophysectomy. Bouin-fixation and haemalum-eosin staining. (× 100.)



TEXT-FIG. 18

TEXT-FIG. 19

TEXT-FIG. 18. *Rana esculenta*. Cross-section through Wolffian duct of a control frog, fixed 7 July 1949. Bouin-fixation and haemalum-eosin staining. (× 100.)

TEXT-FIG. 19. *Rana esculenta*. Cross-section through Wolffian duct of an experimental frog, fixed 7 July 1949, 9 months after hypophysectomy. Bouin fixation and haemalum-eosin staining. (× 100.)

In the experimentals, however, the Wolffian ducts show distinct changes after hypophysectomy; their diameters decrease, while their epithelium becomes low and loses the folds of the normal duct (Text-fig. 19). The regression is visible as early as 2 weeks after the operation and it continues until the end of April. From then on the situation remains stationary.

Again the question arises whether the pituitary acts on the Wolffian ducts directly or indirectly via the testes. As the Wolffian ducts in *R. temporaria*

regress after castration, it is possible that in green frogs the influence of the pituitary acts via the testes. But here, too, the cytological investigation of the interstitial cells does not give any indication. For in our control animals the interstitial cells undergo a strong regression from March till May, whereas the Wolffian ducts do not regress either during or after this period. That both interstitial tissue and the epithelium of the Wolffian ducts show regression after hypophysectomy might, therefore, depend on no causal relation between these two tissues.

Discussion

As we have seen, the prespermatogenetic activity, which in *R. esculenta* occurs in normal animals from October to January, is not interfered with by hypophysectomy (Text-fig. 1). The impression is gained that after ablation of the pituitary the mitotic activity of the primary spermatogonia is arrested and that only their descendants, present at the time of operation, develop further till they have all passed over into primary spermatocytes. They then degenerate rapidly. Consequently, only the primary spermatogonia survive, and 8 months after hypophysectomy all other spermatogenetic cell stages have disappeared from the testis tubules. The latter phenomenon has also been mentioned by Gallien (1940) in hypophysectomized grass frogs.

It is therefore not improbable that only one link in the process of spermatogenesis, viz. the division of the primary spermatogonia, is conditioned by the pituitary.

According to Champy (1913) the number of spermatogonia that divide under natural conditions during the winter months is very small. Whether this phenomenon depends on a shortage of pituitary-hormone or on a non-susceptibility of the spermatogonia for the gonadotropic hormone is not easy to decide. On the other hand, spermatogonial divisions can easily be effected during the winter, provided the pituitary is present. Frogs kept under laboratory conditions (room temperature and plenty of food), possess testis tubules showing strong mitotic activity. In these many cell nests arise (Text-fig. 1), although these processes do not lead to the formation of spermatozoa. Under natural conditions, however, spermatogenesis does not begin before June, but spermatozoa are already present in July (Champy, 1913). Consequently, in the laboratory the gonadotropic influence of the pituitary does not seem to be sufficient to stimulate complete spermatogenesis.

The disappearance of the winter spermatozoa from the testis occurs in the laboratory at the same time that copulations are performed in the field, although in the laboratory copulations cannot take place. After hypophysectomy the spermatozoa also disappear, and it appears probable that this phenomenon is due to the limited duration of life of the spermatozoa, which are absorbed 7–10 months after having been formed.

In the foregoing pages (pp. 137 and 138) it has been shown that during a decrease in spermatogenesis, under normal (Champy, 1913) and under labora-

tory conditions, there is an increase in the mitotic activity of the interstitial cells, and vice versa. Moreover, the absence of the pituitary does not inhibit the multiplication of the interstitial cells. Therefore the impression is gained that the spermatogenetic and interstitial tissues compete with each other for room and for the food substances supplied by the blood. This explains also the cyclic alternation in the development of both tissues under natural conditions.

The function of the interstitial cells depends on the pituitary. Under normal as well as under laboratory conditions an increase in number of these cells is accompanied by an increase in function (Text-figs. 6 and 7). But following hypophysectomy (cf. Text-figs. 14 and 15) the number of cells increases, whereas their volume, and therefore also their function, decreases. This decrease, however, does not fall below a certain threshold in *R. esculenta*, for the interstitial cells retain their large typical nuclei at least 9 months after hypophysectomy.

Therefore it is concluded that in *R. esculenta* the pituitary promotes and maintains only the functional activity and not the mitotic activity of the interstitial cells.

It is probable that in *Rana*, as in the higher vertebrates, the interstitial cells produce a hormone by which, for example, oestrus, the sex characters, and partly also spermatogenesis are influenced.

According to Champy (1913), however, the activity of the interstitial cells in *R. esculenta* is not increased either before or during the copulation period, and it alternates with the spermatogenetic activity. The same alternation was stated in *R. esculenta* under laboratory conditions (p. 137). In *R. temporaria* the short period during which the interstitial cells are active actually occurs shortly after the copulation period. Therefore in both *Rana*-species it is not possible from the data of the normal cycle to establish an endocrine activity of the interstitial tissue on oestrus or spermatogenesis.

As to the sex characters, the thumb-pads, and the Wolffian ducts, it is likely that these are conditioned by the interstitial cells. The thumb-pads of *R. esculenta* resume their development in the period between copulation and spermatogenesis, that is, when the interstitial cells are still very active (Champy, 1913; Harms, 1926). Moreover, an immediate reduction of the thumb-pads and seminal vesicles occurs after castration (Harms, 1926).

We have seen that the same phenomenon takes place after hypophysectomy. Therefore an indirect influence of the pituitary on the sex characters, acting via the interstitial cells, is not impossible. But as we have argued above (p. 140) the cytological changes experimentally induced in the interstitial cells do not point to this action.

De Allende (1939) has demonstrated that in toads, *Bufo arenarum*, the pituitary has a direct influence on the development of the oviduct. Therefore it might be possible that in *Rana* sex characters like thumb-pads and Wolffian ducts are also directly dependent on the pituitary. If this were true, it would present another example of double insurance by which the development of these sex characters is directly influenced by both pituitary and gonads.

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The Structure and Development of the Larval Cuticle of *Diataraxia oleracea* (Lepidoptera)

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With three Plates

Summary

The main layers comprising the soft cuticle of the *Diataraxia* larva have been defined and a study made of their formation during development of the 5th instar larval cuticle.

In soft cuticle the epicuticle is thrown into minute tubercles and consists of three layers—the cuticulin layer, the wax layer, and the cement layer. The cement layer lies outside or partly embedded in the wax layer, but it is either absent or extremely thin over the tips of the tubercles. A polyphenol layer is absent and it is suggested that this layer is present only in hard cuticle.

Beneath the epicuticle is a thin, lightly tanned exocuticle approximately 0·5μ thick.

The 7–10μ thick outer endocuticle is perforated by well-defined pore canals and consists of lamellae containing chitin fibres varying from 100 to 2,000 Å in diameter. Pore canals are absent in the inner endocuticle which when fully developed may be more than 50μ in thickness.

At 25° C., moulting of the 4th–5th instar takes less than 48 hours. The cuticulin layer is laid down about 12–24 hours before ecdysis. At about 6–12 hours before ecdysis the exocuticle is formed beneath the cuticulin layer in the outer part of the developing outer endocuticle. Formation of the wax layer begins about 1 hour before ecdysis, while the cement layer is secreted only a few minutes prior to ecdysis. The inner endocuticle is laid down during the 5 days after ecdysis.

Prior to ecdysis the pore canals connect the hypodermis to the epicuticle and open into the exuvial cavity at the tips of the epicuticular tubercles. Probably the pore canal lumen is responsible for conduction of the moulting fluid.

Each pore canal contains a well-defined strand of material which occupies but a small part of the pore canal lumen. The pore canal strands conduct protein and dihydroxyphenol from the hypodermis to form the exocuticle. Exocuticle formation is complete by about 1½ days after ecdysis. At this stage the pore canal strands become chitinized and sclerotized and the pore canals are cut off from the hypodermis by the developing inner endocuticle. The pore canals are not responsible for transport of regenerative waxes from the hypodermis after abrasion of the epicuticle.

In areas of hard cuticle there is a relatively thick and heavily tanned exocuticle which continues to develop during the life of the instar. The pore canals maintain contact with the hypodermis throughout the instar and probably remain functional as a conducting system for transport of exocuticular materials.

A study of Verson's glands suggested that the large gland cell secretes a lipoprotein which is discharged on the surface of the epicuticle to form the cement layer. The intercalary cell secretes a phenol which is responsible for the tanning of a plug that blocks the opening of the gland duct after discharge.

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Introduction

RECENT studies have shown that the insect cuticle is complex in nature. The innermost part, the endocuticle, consists of a delicate association of protein and chitin (Fraenkel and Rudall, 1947), though in many insects tanning of the protein by quinones results in the formation of a hard inelastic exocuticle (Pryor, 1940*b*) in which the chitin possibly ceases to be of structural significance. Much attention has been paid to the thin superficial epicuticle which has been shown to consist of tanned protein, lipoprotein, and waxes in layers that may be sub-microscopic in size (Wigglesworth, 1947).

However, many biological details still remain to be solved. There appears to be considerable diversity of cuticle structure in different insects and also in different parts of the same insect. Variation in the epicuticle is particularly noticeable: five constituent layers have been demonstrated in *Eomenacanthus* (Webb, 1947), four in *Rhodnius* (Wigglesworth, 1947), and two in *Sarcophaga* (Dennell, 1946). As regards the pore canals it is generally agreed that they act as a conducting system from the hypodermis to the outermost layers of the cuticle and it has been proved that they are responsible for transport of dihydroxyphenols concerned in the formation of hard cuticle (Wigglesworth, 1947, 1948). Otherwise there is little or no evidence to support the various suggestions that they take part in the formation of endocuticle and are responsible for transport of enzymes, proteins, and waxes. The relationship of the pore canals to the epicuticle is uncertain, though in the mature cuticle they appear to end beneath it (Richards and Anderson, 1942; Dennell, 1946) and yet in the developing cuticle they open on the surface (Wigglesworth, 1947, 1948). The properties of the pore canals in the mature cuticle are possibly of importance in connexion with problems of permeability (Richards and Anderson, 1942). In some insects they are connected to the hypodermis throughout life (Wigglesworth, 1933; Richards and Anderson, 1942), whilst in others they are present only in the outer endocuticle (Plotnikow, 1904; Dennell, 1946). Their contents may become chitinized (Dennell, 1946) or sclerotized (Wigglesworth, 1948), or may consist merely of a liquid (Richards and Anderson, 1942).

In the present study particular attention has been paid to the epicuticle and pore canals and an attempt has been made to determine the reasons for what appears to be considerable diversity in their structure and properties in different insects. The tomato-moth larva—*Diataraxia oleracea* L.—was selected for study because many data have been obtained on its resistance to insecticides and it was felt that a knowledge of the cuticle structure was required before problems of permeability could be undertaken. Moreover, *Diataraxia*, which is a Noctuid species (family *Agrotidae*), belongs to the economically important group of plant-feeding insects the cuticles of which have not been studied in the light of present knowledge.

A preliminary examination of the *Diataraxia* cuticle showed a number of interesting features. Although the cuticle is soft there are hardened areas, the

study of which has provided valuable information on the differences between hard and soft cuticle. The dermal glands are relatively large, and in view of the recent findings of Wigglesworth (1947, 1948), who showed that in *Rhodnius* and *Tenebrio* they were responsible for secretion of the cement layer, it was felt that they deserved detailed study in the *Diataraxia* larva.

Material and Methods

Valuable information on the cuticle structure was obtained from the cast exuviae and from strippings of the various layers which were examined in surface view in both the light and electron microscopes. For the latter, special methods of preparation and staining were required. These will be mentioned in the text.

Hand, frozen, and paraffin sections were made, Baker's embedding method being generally used for the frozen preparations and Peterfi's celloidin-paraffin (58° C. m.p. wax) method for the paraffin preparations. For the preparation of serial sections ester wax (Steedman, 1947) and ceresin wax were less effective than the celloidin-paraffin, though the former was useful for cutting individual sections of 0·5μ or less for examination in the electron microscope.

Carnoy and Bouin made good general fixatives, but as found by Dennell (1946), Flemming without acetic was unsurpassed for determining details of the pore canals and epicuticle. Baker's formaldehyde was used for lipoid fixation. With regard to stains, preliminary experiments showed that Mallory's triple stain and Heidenhain's iron haematoxylin gave better results than Mann's methyl blue or Delafield's haematoxylin. Use was made of histochemical tests, details of which will be given appropriately in the text.

Except when otherwise stated the observations were made on 5th instar larvae weighing 0·3–0·6 gm. (3–5 days old at 25° C.). The larvae were fed with cabbage foliage and generally had only 5 instars compared with 6–7 when reared on tomato. At 25° C. the 5th instar lasts 10 days, of which the first 6 days are spent in feeding and the last 4 days in formation of the cocoon and as a prepupa.

The Epicuticle

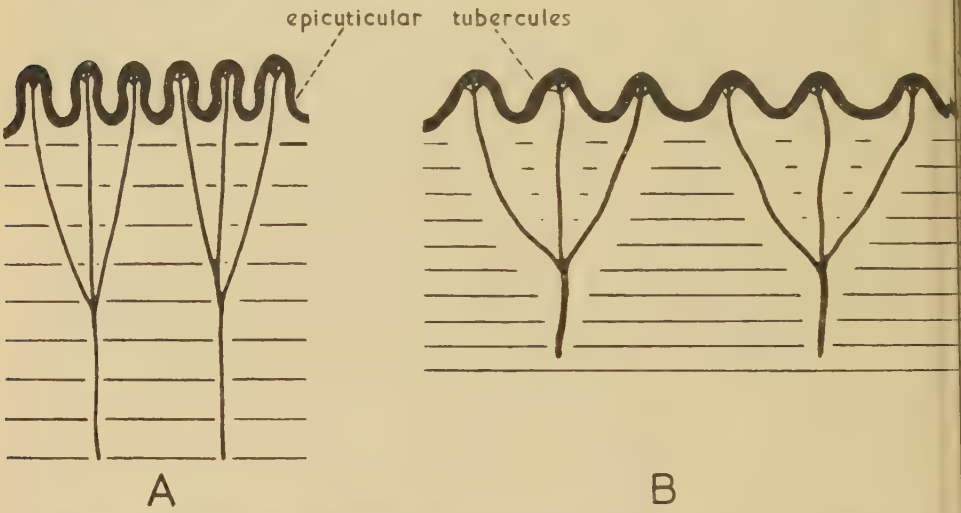
Transverse sections of cuticle, 2μ thick, examined under the light microscope show that the epicuticle is thrown into minute tubercles which are somewhat pointed on the dorsal body-wall but are more dome-shaped ventrally. Electron micrographs of these sections show details of the outlines of the epicuticular tubercles (Pl. I, fig. 1). Dorsally these are about 1·5μ high and about 1μ in diameter at the base, though in the mid-ventral line between the prolegs they may be 3μ high and are readily visible in the light microscope. The tubercles are present in most lepidopterous larvae, and in the earthworm somewhat similar but smaller tubercles (about 600 Å diameter) are present (Reed and Rudall, 1948).

In *Diataraxia* the tubercles cause the surface area of the epicuticle to be 1·5–2 times greater than that of a similar but flat surface, a factor which must decrease efficiency of water conservation. However, this is not a problem for

most phytophagous insects, though it is significant that in *Ephestia* where water conservation is important the tubercles are absent or much reduced. The effect of the tubercles on the surface-active properties of the epicuticle must be considerable, a problem of importance in connexion with adhesion of insecticidal sprays and dusts.

Function of the Tubercles

Tubercles of this type apparently occur exclusively in soft cuticle and it seems probable that they are associated with the mode of life of the soft-bodied animal. In the first place they would improve flexibility of the epicuticle, and secondly they probably act in much the same way as the stellate



Text-fig. 1. Schematic transverse sections of epicuticle and outer endocuticle on the ventral surface of the *Diataraxia* larva showing the effect of cuticle growth. A, new cuticle just prior to ecdysis. B, mature cuticle 4–5 days after ecdysis.

folds of the *Rhodnius* nymphal cuticle (Wigglesworth, 1933) and allow extension and growth of the endocuticle without placing undue strain on the relatively inextensible epicuticle. The effect of growth on the epicuticle may be shown by comparing transverse sections of young and old cuticles. In the newly moulted 5th instar larva the tubercles are pressed together with their bases indented whilst in the mature larva the bases are pulled out (Text-fig. 1). Thus the increase in actual surface area with larval growth is relatively small and consequently the formula $surface\ area = weight^{\frac{2}{3}} \times K (= constant)$ which has been used for surface area determinations of approximately cylindrical-shaped insects is liable to considerable error when applied to lepidopterous larvae.

Structure of the Diataraxia *epicuticle*

Both paraffin and hand sections of unfixed cuticle show an epicuticle which stains grey with sudan black B, whilst the endocuticle remains unstained.

Perhaps this indicates the presence of lipoid. However, it was of interest to carry out the test described by Wigglesworth (1947) which demonstrated the presence of bound lipoid in the cuticulin layer of the *Rhodnius* cuticle. Cast exuviae of *Diataraxia* larvae were boiled with chloroform to remove free lipoid. An exuvium was then gently warmed with chlorinated nitric acid. Initially there was considerable effervescence on the inner surface of the exuvium leaving a thin transparent membrane which on further heating broke down with the liberation of numerous oily droplets. If the heating is stopped just before liberation of the droplets the membrane stains readily in sudan black B. Similar treatment with 10 per cent. caustic potash also causes dissolution of the inner layer leaving a thin membrane which fuses and finally breaks down into large numbers of oily droplets.

Thus, in its response to chlorinated nitric acid the exuvium of *Diataraxia* behaves as does the *Rhodnius* exuvium. The thin transparent membrane is clearly equivalent to the cuticulin layer of *Rhodnius* which Wigglesworth (1947) suggests is composed of a tanned lipoprotein. Now this membrane which forms the outer surface of the exuvium is insoluble in cold caustic potash and cold concentrated hydrochloric acid. It shows no response to the ninhydrin reagent but stains strongly in sudan black B after gentle warming in acid. In the possession of these properties it resembles the outer epicuticle of the *Sarcophaga* larva (Dennell, 1946) except that the latter stains in sudan black B without prior acid treatment. It would appear that the cuticulin layer and the outer epicuticle are synonymous.

Prior to ecdysis the endocuticle of soft cuticle is completely digested by the moulting fluid, leaving a very thin exuvium. However, when the exuvium is warmed with chlorinated nitric acid, the initial copious effervescence from the inner surface suggests that this membrane does not consist entirely of the cuticulin layer. Further information was obtained by treating portions of cuticle with concentrated hydrochloric acid and then sectioning and staining with Mallory. In the untreated cuticle the outer region, which ultimately becomes the exuvium, stained bright red, but after treatment in the cold for 2 hours or warming for a few minutes it stained only faintly or not at all. With the light microscope it was not possible to determine any decrease in thickness after acid treatment of the approximately $0{\cdot}5\mu$ thick exuvium, but the evidence as a whole suggests that acid treatment removes a layer of basic staining material from the inner surface.

Electron micrographs of the exuvium before and after acid and alkali treatment provide clear evidence of the presence of an inner layer. If a portion of exuvium is examined in the electron microscope the exuvium is sufficiently transparent for some detail to be observed at 100 k.v. At 50 k.v. electron scattering is so considerable that little or no detail is visible. Treatment of portions of exuvium with cold 5 per cent. hydrochloric acid, 5 per cent. caustic potash, or diaphanol for 2–3 days, or with 1 per cent. pepsin overnight causes relatively little increase in transparency of the membrane framework to the electron beam. This suggests that the inner layer is not soluble in the above reagents at

the temperature and during the period of treatment stated. However, treatment of cuticle or exuvium with cold concentrated hydrochloric acid overnight removes the inner layer, leaving the cuticulin layer which is now so thin (probably about 0·1–0·2μ thick) that it is almost transparent even at 50 k.v.

In its properties the inner layer of the exuvium, although very thin, resembles the exocuticle which hitherto has been said to occur only in hard cuticle. However, it also has properties in common with the inner epicuticle of *Sarcophaga* (Dennell, 1946). Now a thin exocuticle and an inner epicuticle would occupy a similar position in the cuticle, the only difference being that the former, which is laid down in the outer endocuticle, contains a proportion of chitin in addition to tanned protein, while the latter apparently consists solely of protein or tanned protein. Further data on the nature of this layer will be given later.

Structural detail on the outer surface of the cuticulin layer was determined by a process of shadowing whereby a thin layer of metal cast at an angle to the surface of the preparation was used to throw all irregularities on the surface into highlights and shadows. A portion of newly formed dorsal cuticle was treated with concentrated hydrochloric acid overnight to remove all layers except the cuticulin layer which was then shadowed with nickel and examined in surface view in the electron microscope (Pl. I, fig. 2). Clearly the cuticulin layer is continuous even over the surface of the tubercles, though there are indications of a pore at the tip. Pl. I, fig. 2, shows that removal of the supporting inner layer by the acid has resulted in collapse of the tubercle which has fallen on its side. Otherwise there is no sign that the cuticulin layer has been affected by the acid treatment.

Analysis of the Exuvium of Soft Cuticle

Apart from excreta and remnants of moulting fluid the *Diataraxia* exuvium consists solely of those parts of the cuticle which are not digested by the moulting fluid, namely, the exocuticle and the epicuticle. Thus, it provides valuable material for studying the composition on these layers. Prolonged treatment of the exuvium with cold concentrated hydrochloric acid removes all constituents except the cuticulin layer and the wax layer. Similar treatment with 20 per cent. caustic potash leaves the chitin in addition to the cuticulin and wax layers. Such treatments can be used to determine the proportions of the various constituents of the exuvium.

Cast exuviae of 5th instar larvae were divided into batches of about 100. The head capsules were removed because these consist almost entirely of hardened exocuticle. The remaining body cuticle exuviae were ground coarsely and then washed at 60° C. for about 8 hours with several changes of water. This treatment causes a weight loss of about 15–20 per cent. which probably represents unabsorbed moulting fluid and excreta.

It should be made clear that the proportions of the various constituents will be expressed as percentages by weight of the exuvium *after* removal of water-soluble material. Materials soluble in the moulting fluid which are probably

present in the living epicuticle and exocuticle are not included in the present analysis, in which only the exocuticular chitin, tanned protein, lipoprotein, and wax constituents are considered.

The batches of exuviae were treated as follows:

1. With 20 per cent. caustic potash at 25° C. Treatment was carried out in platinum crucibles and filtration and washing in Gooch filters.
2. With concentrated hydrochloric acid at 25° C. Treatment throughout was carried out in Gooch filters.

3 and 4. As 1 and 2, except previously extracted with chloroform at 60° C., the weight of the chloroform extracts (i.e. the wax layer) being determined.

Twenty-four hours after treatment, and at 2-day intervals afterwards, the residue was washed thoroughly with water, dried at 60° C. over phosphorus pentoxide, and weighed until constancy was reached. This occurred in all treatments after 5–7 days.

The percentage weights of residue remaining after treatment are given in Table I.

TABLE I. *Weights of Residue remaining after Treatment of Last Instar Exuviae with 20 per cent. Caustic Potash and Concentrated Hydrochloric Acid*

Treatment	*Wts. of residues as per cent. of exuvium*
20 per cent. caustic potash	31·9
Chloroform extracted + 20 per cent. caustic potash	25·4
Concentrated HCl	19·5
Chloroform extracted + concentrated HCl	13·3

The wax extracted by the chloroform amounts to 4·7–5·2 per cent. of the exuvium, a figure which agrees closely with the 4–4·5 per cent. obtained by Bergmann (1938) for silkworm exuviae, particularly when it is remembered that the latter determination was made on exuviae which had not been extracted with water and thus contained a higher proportion of non-lipoid material. Table I shows that in both acid and alkali treatments the reduction in weight of residue after chloroform extraction of exuviae is just over 6 per cent. This reduction may be accounted for by the absence of the wax layer, which at 25° C. is not dissociated by either acid or alkali.

The residue after acid treatment consists of the cuticulin layer, while that remaining after alkali treatment contains chitin in addition. The difference in weight gives the proportion of chitin which therefore represents about 12 per cent. of the exuvium. Thus, proportions of exuvium constituents are: wax 5 per cent., cuticulin 13 per cent., chitin 12 per cent., other constituents (mainly protein) 70 per cent.

The chitin content corresponds with the 10–20 per cent. present in the silkworm exuvium (Kuwana, 1933; Bergmann, 1938). To confirm the chitin figure, chloroform-extracted exuviae were treated with 20 per cent. caustic

potash at 60° C. for 2 days. The residue which had not reached constant weight was 19·2 per cent. of the original. It was then immersed in cold concentrated hydrochloric acid for 1 day to hydrolyse the chitin and a residue of 6·8 per cent. was left. The difference of 12·4 per cent. confirmed the figure for chitin obtained above, but the low figure for the residue of cuticulin shows that this material is dissociated by caustic alkali at 60° C. However, destruction is slow because remnants still remain even after treatment for 1 week. The cuticulin layer is also slowly dissociated by concentrated hydrochloric acid at 60° C.

Nitrogen Content of Exuvium

To obtain some data on protein content, nitrogen determinations of the exuvium and of the exuvial residues after acid treatment were made by the micro-Kjeldahl technique. Table II shows the figures obtained for percentages of nitrogen.

TABLE II. *Nitrogen Contents of Exuvium and of Residues after Acid Treatment*

Treatment	*Per cent. nitrogen*
Exuvium after washing with water	11·2
Conc. HCl at 25° C.	4·1
Chloroform at 60° C.+conc. HCl at 25° C.	5·9

The residue after hydrochloric acid treatment consists of cuticulin layer plus wax layer. If the latter is discounted on the basis that it contains no nitrogen, the calculated nitrogen figure for the cuticulin layer is 5·7 per cent. which agrees with the 5·9 per cent. content of the residue after chloroform plus hydrochloric acid treatment.

Since there is no evidence to suggest that the nitrogen represents anything but protein and chitin, approximate figures for percentages of protein in the cuticulin layer and in the inner layer of the exuvium may be obtained by multiplying the nitrogen figure by 6·25 after allowance has been made for the nitrogen present as chitin (theoretical figure = 6·9 per cent.) and the 0 per cent. nitrogen in the wax layer.

The calculated percentages of the exuvium constituents with nitrogen and protein contents are given in Table III.

TABLE III. *Percentages and Protein Contents of Exuvium Constituents*

	Per cent. present in exuvium	*Per cent. nitrogen*	*Per cent. $N_2 \times 6{\cdot}25$ (per cent. protein)*
Wax layer	5	..	..
Cuticulin layer	13	5·9	36·9
Inner layer:			
Chitin	12	6·9 (theoretical)	..
Other constituents (mainly protein)	70	13·7	85·6

As will be shown later the figure of 70 per cent. for other constituents includes the cement layer which, however, must be present in very small proportion. Because of this it is probable that the correct figure for the proportion of non-chitinous constituents of the inner layer is slightly lower than that shown.

The evidence in Table III suggests that the inner layer of the exuvium contains chitin and thus is an exocuticle and not an inner epicuticle. From the table it can be calculated that the exocuticle contains about 15 per cent. chitin and about 73 per cent. protein. Tanning materials and pigments would be included in the remaining 12 per cent.

As previously mentioned the above figures are not strictly accurate, but they demonstrate that the cuticulin layer contains protein in addition to lipoid and that the inner layer is a thin exocuticle. If it is assumed that the cuticulin layer consists solely of lipoprotein, the lipoid content is in the region of 60 per cent. This would account for the copious evolution of oily droplets which occurs when the cuticulin layer is dissociated with hot caustic potash or chlorinated nitric acid.

The Wax Layer

For a number of insect species Wigglesworth (1945) and Beament (1945) have shown that a thin layer of orientated wax on the surface of the epicuticle is responsible for waterproofing. Abrasion or treatment with a wax solvent disorganizes the wax layer and increases permeability of the cuticle. Table IV shows the results of such treatments with batches of mature last instar larvae of *Diataraxia* as test-subjects. All larvae were killed in ammonia and the spiracles, head, and anus blocked with celloidin before determination of transpiration was carried out.

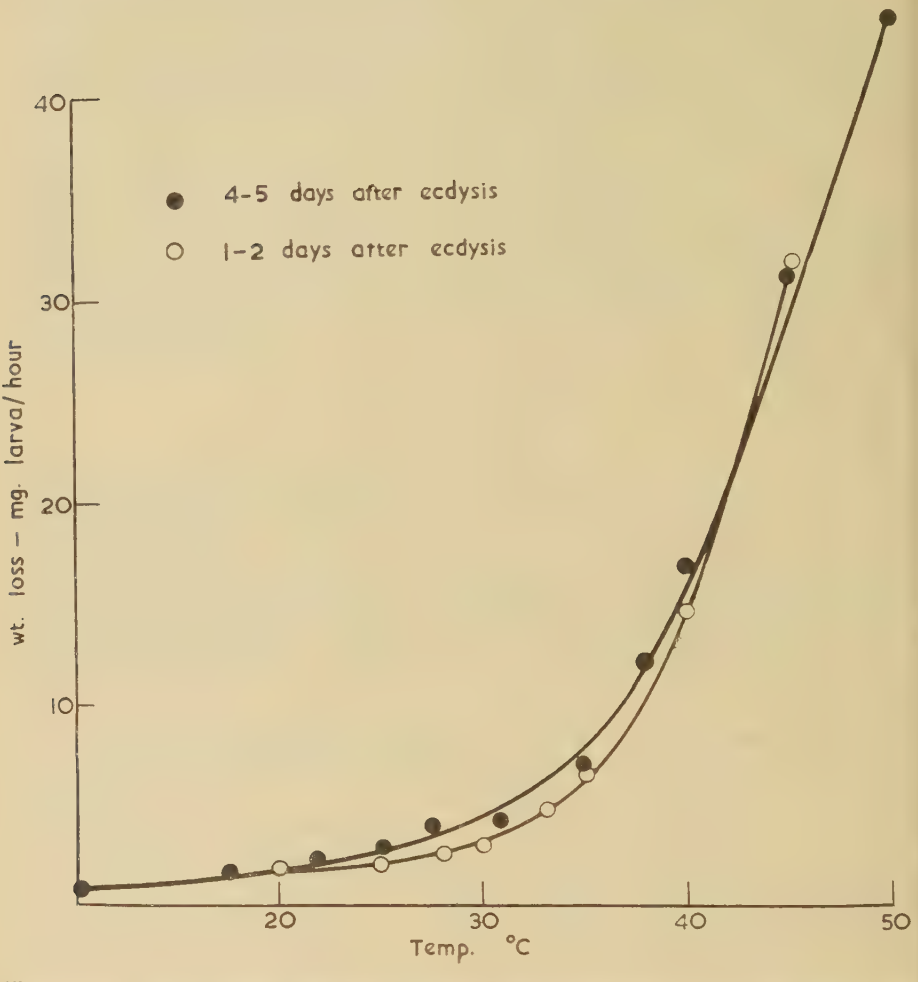
Table IV. *Water Loss of the Cuticle in Dry Air at 25° C. after Abrasions and Chloroform Treatment*

Treatment	*Water loss mg./larva/hour*
Small area of dorsal surface gently abraded with alumina	10·2
,, ,, ,, harshly ,, ,,	12·6
Larvae immersed in cold chloroform ½ minute	10·8
Larvae suspended over chloroform ¼ hour	3·5
Larvae untreated	2·8

Wigglesworth has shown that removal of the wax layer exposes material which stains readily when the insect is immersed in ammoniacal silver hydroxide. In the mature *Diataraxia* larva such staining is never intense but consists of a light-red colour in the framework of the epicuticle. It is interesting that gentle abrasion often does not expose silver staining areas, though the increased permeability which results may be almost as great as that caused by harsh abrasion (see Table IV). Clearly the properties of waterproofing reside in the superficial layers and not in the framework of the epicuticle.

Observations of Ramsay (1935), Wigglesworth (1945), and Beament (1945) show that at a particular temperature—the critical temperature—the wax

layer undergoes a change of phase and allows water to pass readily through the cuticle. Text-fig. 2 shows graphically the weight losses of last-instar *Diataraxia* larvae in mg. per larva per hour in dry air at various temperatures. Two sets of



TEXT-FIG. 2. Graph showing relationship of water loss to temperature for the last instar larva of *Diataraxia*.

data are shown, one with 1- to 2-day-old larvae and the other with 4- to 5-day-old larvae. Both show that the critical temperature lies in the region of 31–34° C. For comparison the critical temperature obtained by Wigglesworth (1945) for other larvae living in a similar habitat are: *Nematus*, *c.* 35° C.; *Pieris*, *c.* 42° C. Thus the waterproofing mechanism in the *Diataraxia* larval cuticle is essentially similar to that of the range of insect species examined by Wigglesworth (1945) and Beament (1945).

Data already described show that the chloroform-soluble material of the

Diataraxia larva exuvium, which is considered to form the wax layer, constitutes about 5 per cent. by weight of the exuvium or about 0·47 per cent. of the dry weight of the complete 5-day-old cuticle.

The Cement Layer

Paraffin sections of soft cuticle sometimes show a thin layer of material which has separated or partly separated from the outer surface of the epicuticle. It has the appearance of a network somewhat like a minute sheet of chicken wire which when in position lies in the depressions surrounding the epicuticular tubercles. The tubercles themselves project through the holes in the network. The layer can be demonstrated in sections of abraded cuticle, when it is seen that abrasion has torn away short lengths of the layer which remains attached here and there to the body of the epicuticle. The layer is remarkably thin and difficult to see, particularly since it does not stain with acid dyes, and with basic dyes stains only very weakly in comparison with the epicuticle. In paraffin sections it stains a dark grey with sudan black B, though it is perhaps most readily demonstrated by its reddish colour after chloroform treatment or abrasion followed by immersion in ammoniacal silver oxide (Pl. II, fig. 15).

The ease with which this layer can be separated from the epicuticle by abrasion and chloroform treatment suggests that it is not strongly bound. It occupies the position of the cement layer as described by Wigglesworth (1947, 1948) for *Rhodnius* and *Tenebrio* since it lies on the outside of the cuticle and is separated from the cuticulin layer by a layer of chloroform soluble material—presumably the wax layer.

A sheet of larval cuticle freed from all underlying cellular material was fixed in Carnoy and stuck firmly to a glass slide, endocuticle downwards. The preparation was dried on a hot plate and then a thin layer of collodion poured over the surface of the epicuticle and allowed to dry. Successive layers were poured on and dried until a tough membrane was obtained which was then peeled away, carrying with it the cement layer. It was laid on a fresh slide, allowed to dry, and the celloidin was then dissolved away in acetone. The remaining cement layer was deeply stained with sudan black B or iron haematoxylin and examined in the light microscope (Pl. I, fig. 3).

As far as can be determined the cement layer covers the whole body. Its presence was demonstrated on the hard cuticle of the head and muscle insertions over the flat surface of which it forms a thin continuous covering (Pl. I, figs. 3 and 5). Variations in intensity of staining suggest differences in thickness which give the impression that the layer has been formed by flowing as a liquid over the surface of the epicuticle and setting somewhat unevenly.

Further details were obtained by examination in the electron microscope. It was found that collodion-stripped preparations were generally contaminated with impurities in the collodion (Pl. I, fig. 5), and the best material was obtained by warming portions of the cuticle in chloroform. Pieces of cement layer sufficiently large for examination in the electron microscope could then

be scraped off with a fine needle. These were mounted on microscope grids without supporting collodion film and examined in surface view (Pl. I, fig. 4). The layer is probably not more than 0·1μ thick as observed in the electron microscope. In limited areas, generally on the ventral body-wall and in the neighbourhood of the muscle insertions (Pl. I, fig. 5), it sometimes covers the tubercles, but elsewhere the tips of the tubercles project through it, though if the layer here is extremely thin it might be torn away during the stripping process. Nevertheless it is difficult to assign a protective function to this layer since it affords either little or no protection to those areas of the cuticle which are most likely to become abraded. Apparently the cement layer is partly embedded in the wax layer. Thus although it does not provide protection, it may act as a supporting framework to the wax layer.

In electron micrographs of intact exuvium the cement layer is seldom visible, probably because it causes insufficient electron scattering to contrast against the diffused scattering of the approximately 0·5μ thick exocuticle. Examination of the epicuticle after overnight treatment with cold concentrated hydrochloric acid or 20 per cent. caustic potash shows no sign of it. This suggests that the cement layer is dissolved by concentrated acids and alkalis, an observation which was confirmed by experiments in which drops of concentrated acid and alkali were placed for 1 hour on portions of fresh cuticle, which were then fixed in Carnoy and subjected to the collodion stripping process. Strippings from the treated areas did not contain a cement layer. However, in preparations where only the undersurface of a portion of exuvium was treated for 2–3 hours with concentrated hydrochloric acid the greater part of the exocuticle was removed, leaving the cement layer intact and clearly visible in electron micrographs (Pl. I, fig. 6).

The cement layer cannot be removed from the cuticle without prior treatment with a wax solvent, and thus it was not possible to carry out histochemical tests on fresh material. Portions of cement layer removed from Carnoy-fixed cuticle were soluble in warm concentrated hydrochloric acid and fused into a yellowish mass and finally dissolved when warmed with 20 per cent. caustic potash. A few oily droplets were observed when the cement layer dissolved in potash. The response to ninhydrin was negative, though even after Carnoy treatment the cement layer stained grey with sudan black B. Doubtful significance is attached to the above observations, though they suggest that the cement layer of *Diataraxia* has properties somewhat similar to the cement layer of *Rhodnius* and *Tenebrio* (Wigglesworth, 1947, 1948).

Pore Canals in the Epicuticle

Sections of the *Diataraxia* cuticle show that a single pore canal runs inside each epicuticular tubercle. Otherwise no further detail can be determined under the light microscope because structures within the tubercles are too small for resolution. Portions of soft exuvium examined in surface view in the electron microscope show no indications of pore canal openings, but if the exuvium is treated with any of the following: (1) diaphanol for 3 days; (2)

5 per cent. caustic potash or 5 per cent. hydrochloric acid overnight; (3) 1 per cent. pepsin at pH 2 and 32° C., an aperture is disclosed at the tip of each epicuticular tubercle (Pl. I, fig. 7). This undoubtedly is the external opening of the pore canal. The apertures are also disclosed after treatment with concentrated mineral acids and concentrated alkalis which also remove the exocuticle. Clearly the pore canals pass through both exocuticle and epicuticle, but in the mature epicuticle they appear to be blocked by a plug of material which is fairly easily dissolved or dislodged. Evidence for the presence of the plug is given in preparations of the cement layer by the collodion stripping process which removes a lump of material from the tip of each tubercle (Pl. I, fig. 5). Moreover, the small irregular swellings at the tips of the tubercles, as shown in electron micrographs of the dorsal cuticle (Pl. I, fig. 1), suggest a cap of secreted material. Perhaps the plugging of the pore canals is responsible for their not being observed in the mature larval epicuticle of *Sarcophaga* (Dennell, 1946) and the adult epicuticle of *Periplaneta* (Richards and Anderson, 1942).

Data on the penetration of water-soluble stains such as ammoniacal silver hydroxide which will be given later suggest that during the pre-ecdysial period the pore canals are open and provide a channel for rapid penetration of water-soluble material to the hypodermis. Thus untreated preparations of the newly formed epicuticle should show open pore canals. However, all the preparations made were contaminated with either remnants of hypodermis or newly formed endocuticle and were opaque to the electron beam. The absence of the irregular cap at the tip of the tubercles, and failure to remove it by the stripping technique, support the evidence that the pore canals are open in the newly formed cuticle.

The readiness with which the plug can be removed from the pore canal openings in the mature epicuticle suggests that the pore canals may still provide a relatively easy path for the penetration of water soluble materials, in particular through the lipoprotein cuticulin layer.

Pore Canal Contents and the Epicuticle

Transverse sections of mature cuticle stained in Mallory or iron haematoxylin show that the pore canal contents form well-defined tree-like structures in the outer endocuticle (Pl. I, fig. 8). A single branch runs inside each epicuticular tubercle and appears to make contact with the exocuticle or epicuticle near the tip. Further detail is given by electron micrographs. A portion of ventral cuticle was laid endocuticle downwards on concentrated hydrochloric acid for 10 minutes. The endocuticle was disorganized, leaving the exocuticle intact, and under the high power of the light microscope the pore canal strands were observed as a mass of hair-like processes projecting from the under surface of the exocuticle. After fixation in Carnoy, remnants of the endocuticle were gently brushed away and the remaining membrane examined in surface view in the electron microscope. Pore canal strands were seen passing up and branching over the inner surface of each tubercle (Pl. I, fig. 9). Internally the

strands from adjacent tubercles, 6–12 dorsally and 4–7 ventrally, converge and unite to form a single main strand, the whole forming the tree-like structure observed in the light microscope (Pl. I, fig. 8).

The pore canal strands are not digested by the moulting fluid, and thus remnants usually remain attached to the cast exuvium. Pl. I, fig. 10, is an electron micrograph of a portion of exuvium treated with osmium tetroxide which has acted as an electron stain. This preparation shows clearly that within the tubercle each pore canal strand divides into a number of fine filaments which enter the epicuticle or exocuticle. The ramifications of these filaments within and between the epicuticular tubercles are best seen in more heavily stained preparations and show that each pore canal strand is intimately associated with the outer layers of the cuticle. If the exocuticle is removed by treatment with concentrated hydrochloric acid overnight and the remaining cuticulin layer treated with Altmann and examined in the electron microscope the fine osmium-staining filaments have disappeared. This may suggest that the filaments lie within the exocuticle, but in view of the possible effects of the acid treatment the evidence is unreliable.

Composition of Pore Canal Contents

Strands of material in the pore canals have been observed by a number of authors and were described as consisting of cytoplasmic or cuticular material mainly on the basis of their staining properties. A more detailed study of the pore canal contents of the mature *Sarcophaga* larva (Dennell, 1946) showed that these contained chitin, though in the immature stage they are apparently cytoplasmic. Campbell's (1929) modification of the van Wisselingh test for chitin as described by Dennell (1946) was carried out on the mature larval cuticle of *Diataraxia*. The pore canal strands gave a strong positive response. However, unlike the acidic staining pore canal contents of *Sarcophaga*, the pore canal strands of *Diataraxia* stain strongly with basic dyes. Moreover, the strands are not digested at moulting nor are they destroyed by $\frac{1}{2}$ hour treatment of the fresh cuticle with cold concentrated hydrochloric acid. Treatment of a portion of cuticle with cold concentrated nitric acid for $\frac{1}{2}$ hour followed by fixation with Carnoy, sectioning, staining, and examination under the light microscope shows that the strands have fused into a number of particles which still stain red with Mallory. However, the pore canal strands are completely destroyed by treatment overnight with cold concentrated acid.

In the possession of the above properties the pore canal strands are identical with the exocuticle. Since they are attached to the exocuticle they may be considered as part of this layer, the probable function of which is to support the delicate cuticulin layer and also to act as a bridge between the lipophil cuticulin layer and the lipophobe endocuticle. In the latter respect the pore canal strands must be important because they form exocuticular roots running into the endocuticle.

The Endocuticle

The soft endocuticle of the *Diataraxia* larva is divisible into an outer endocuticle which over most of the body is 7–10μ thick, and an inner endocuticle which may be 50μ or more in thickness. The former shows a somewhat stronger staining response to acidic dyes, though after Flemming fixation it stains readily in iron haematoxylin in contrast with the inner endocuticle. The outer endocuticle also possesses well-defined pore canals containing basic staining pore canal strands (Pl. I, fig. 8) and it is clearly lamellate, whereas the more widely spaced lamellae of the inner endocuticle are less easily defined.

The Outer Endocuticle

After fixation in Flemming, 2μ thick sections of the outer endocuticle were cut parallel to the cuticle surface, stained in iron haematoxylin, and examined in the light microscope. In such preparations the lamellae are seen to be fibrous in nature, though no detail can be determined. On the ventral integument between the prolegs the pore canals are much larger than elsewhere and are well-defined holes sometimes 3μ in diameter in the periphery of the outer endocuticle and becoming somewhat smaller in the deeper lamellae until they disappear completely in the region of the inner endocuticle. In each canal one or more heavily stained dots—the pore canal strands—are visible and it is clear that these occupy but a small part of the pore canal lumen (Pl. I, fig. 11). The difference between outer and inner endocuticle is in part due to the chitin constituents, for if the cuticle is treated overnight with 5 per cent. caustic potash at 100° C. to remove protein and is then sectioned, the inner and outer endocuticles still show differences in their ability to absorb stains. The latter is more retentive of both acidic and basic dyes, and transverse and surface sections stained with iron haematoxylin show fibrous structure and pore canals in which the chitinized pore canal strands are still visible.

The lamellation of the endocuticle is well defined after alkali treatment. This observation contrasts with that of Richards and Anderson (1942) who state that in the *Periplaneta* endocuticle the lamellations disappear after potash treatment, though Yonge (1932) and Dennell (1946) observed that lamellation remained after such treatment of the fore-gut cuticle of *Homarus* and the body cuticle of the *Sarcophaga* larva. Treatment of a portion of *Diataraxia* cuticle with concentrated hydrochloric acid for about 5 minutes followed by fixation and sectioning shows that the lamellations have disappeared. These observations suggest that the chitin is concentrated in sheets and that it is responsible for the lamellations observed.

Fine detail was determined by studying strips peeled from the outer endocuticle which were then examined in surface view in the light and electron microscopes. Prior to being mounted on the electron microscope grid the strips were kept in water for about $\frac{1}{2}$ hour and consequently water-soluble protein was largely removed. The strips were generally too thick, but in places, and in particular at the edges of the preparations, areas one lamella thick were

obtained which were suitable for examination by transmission in the electron microscope. Relatively large sheets of outer endocuticle consisting of single lamellae were readily obtained by dissection from the old cuticle of larvae just prior to moulting. At this stage the old endocuticle is in process of being digested, the initial stages of which result in a softening or destruction of the bonds between individual lamellae. In such preparations it is probable that water-soluble proteins had been removed and possibly some digestion of other constituents had occurred. However, under the electron microscope these preparations showed no difference in structure from the strippings of the mature cuticle and they were therefore used in most of the treatments described below.

Electron micrographs of lamellae from the outer endocuticle show that the pore canals are subdivided (Pl. I, fig. 12). Probably each subdivision contains a single branch of tree-like pore canal strand. Prior to examination the preparation shown in Pl. I, fig. 12, was treated with pepsin to remove protein and thus the micrograph shows only the chitin constituents. However, examined by transmission this lamella and also lamellae treated with diaphanol and caustic potash showed no detectable difference from untreated lamellae. In all preparations little structural detail was observed in the framework of the lamella, though there are indications of the presence of fibres or fibre bundles. These fibres must consist of chitin, though Pl. I, fig. 12, shows that considerable areas of chitin are apparently structureless. However, the impression given by such pictures is incorrect, for if a lamella is metal-shadowed and then examined in the electron microscope, a system of fibres far more complex than is apparent in transmission pictures is visible on the surface of the lamella (Pl. II, fig. 13). Clearly the apparently structureless areas consist of a mass of small chitin fibres orientated at random in the plane of the lamella. The smallest fibres are rather less than 100 Å in diameter, while the largest fibre bundles may be 2,000 Å in diameter. The fibres are readily visible in shadowed preparations of untreated lamellae, but prior treatment with pepsin, diaphanol, or caustic potash causes some improvement in definition. Possibly the better definition is due to removal of water-insoluble protein constituents, but the micrographs were not sufficiently clear to determine any significant difference in the appearance of the fibres.

Richards and Korda (1948) obtained transmission micrographs of a number of insect membranes, including the chitin of the tracheal membrane of *Periplaneta* which consists of fibrils and fibres 100–300 Å in diameter and similar in appearance to those of the outer endocuticle of *Diataraxia*. However, 'unexpected diversity in chitin patterns' was found in other insect membranes, some of which appeared homogeneous. The present work has shown that such transmission micrographs may give a false impression of the structure of chitinous membranes. Rather thick preparations may appear homogeneous until shadowed, when chitin fibrils are clearly revealed. Even thin preparations examined by simple transmission give little indication of the chitin pattern, and it seems possible that the diversity in structure of insect membranes

examined by Richards and Korda (1948) is more an index of their different thicknesses than a variation in chitin cross-linkages.

To summarize: present evidence suggests that the outer endocuticle is made up of about 10 lamellae approximately 1 μ apart. The lamellae are pierced by well-defined pore canals which may be 1–3 μ in diameter in peripheral lamellae but decrease in diameter as the pore canal strands which they surround, converge and combine in the deeper lamellae of the outer endocuticle. Each lamella comprises a sheet of randomly orientated chitin fibres and bundles of fibres presumably associated with water-insoluble and water-soluble proteins. The chitin is responsible for the lamellations whilst protein is the main constituent of the interlamellar region.

Fraenkel and Rudall (1947) described X-ray and swelling data on cuticles of *Sarcophaga* which suggest layers of chitin separated by protein. The present findings are in agreement with this general statement. However, Fraenkel and Rudall suggest that the association implies a model of alternating mono-layers of chitin and protein. This theory is based on the fact that three amino-acid residues occupy almost the same volume as one chitobiose residue and for equal lengths of chitin and protein chains the weight ratio is 55 : 45, which is a close approximation to the 54 : 46 ratio obtained in their determination with the *Sarcophaga* larval cuticle. Table V shows that the 55 : 45 ratio does not occur in other soft-bodied insects so far studied, and present work on development of the *Diataraxia* cuticle shows that the chitin-protein ratio does not approach 55 : 45 at any stage in cuticle growth. Moreover, the *Diataraxia* outer endocuticle consists of approximately 0·5 μ thick lamellae containing mainly chitin separated by layers consisting mainly of protein, and it is difficult to conceive how this structure can be explained in terms of an alternating mono-layer theory.

TABLE V. *Ratios of Chitin and 'Protein' present in the Last Instar Larval Cuticle of Various Lepidoptera*

	Chitin	*'Protein'**
Ephestia elutella . . .	18	82
Plutella maculipennis . .	20	80
Pieris brassicae . . .	35	65
Diataraxia oleracea . .	36	64
Plusia gamma . . .	38	62

* Material soluble in 5 per cent. KOH at 90° C.

Electron micrographs of cellulose from the primary wall of various plant fibres (Mühlethaler, 1949) are remarkably similar to those of the outer endocuticle of *Diataraxia*. In the wall of a plant cell the cellulose chains form visible lamellae (Preston and Astbury, 1937) and—at any rate in the developing primary wall—are associated with protein (Tupper-Carey and Priestley, 1924). The similarity of structure is understandable since both primary cell wall and outer endocuticle require similar specialized properties, notably tensile strength

combined with the ability to undergo considerable stretching, during meristematic activity in the former, and just after ecdysis in the latter.

The Inner Endocuticle

The inner endocuticle is that part of the cuticle which is laid down after moulting. The lamellae are larger and more widely spaced than in the outer endocuticle, a condition similar to that in the soft-bodied *Sarcophaga* larva (Dennell, 1946). In *Diataraxia* this difference is certainly in part due to the effect of growth, because most of the inner endocuticle is laid down after the first day and, unlike the outer endocuticle, is subjected to relatively little stretching and consequent 'thinning' of the lamellae.

In transverse sections under the light microscope the lamellae are not readily distinguished from the interlamellar tissue. However, they separate individually during digestion of the endocuticle at moulting and their fibrous structure is readily visible in the latter stages of this process. Pore canals and pore canal strands are absent in the inner endocuticle, a condition which appears to be typical of soft-bodied larvae (Plotnikow, 1904; Dennell, 1946).

Electron microscope studies have as yet produced little additional information on the structure of the lamellae. Strips of cuticle examined in surface view are rather opaque to the electron beam, partly because the lamellae are thicker and also because they seem to be less readily separated than lamellae of the outer endocuticle. Strippings and transverse sections of the inner epicuticle showed only that large fibre bundles are present and these are larger than those in the outer endocuticle.

Formation of the 5th Instar *Diataraxia* Cuticle

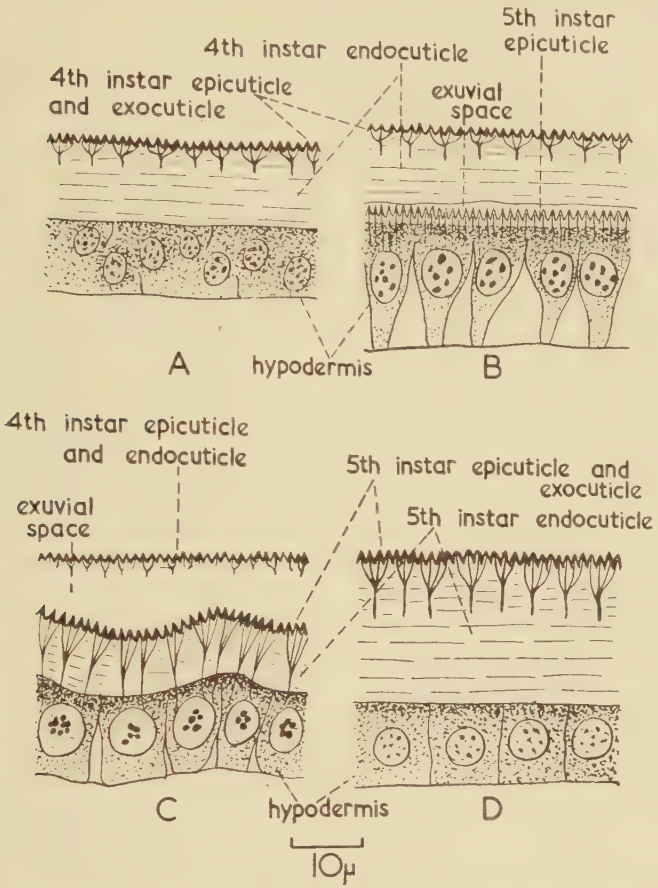
Moulting of the *Diataraxia* larva is a relatively rapid process—the time between cessation of feeding of the 4th instar and emergence of the 5th being rather less than 2 days at 25° C. The time scale for the stages in development cannot be determined exactly owing to rapidity of the various processes which occur mainly in the 12 hours prior to moulting. These processes do not occur simultaneously throughout the body but are initiated in the head region and show an axial gradient (cf. Kühn and Piepho, 1938).

In the cuticle the first visible sign of moulting is about 6–12 hours after cessation of feeding when cell division begins in the hypodermis in an apparently haphazard manner (Text-fig. 3*a*), the cells having no definite shape and the cell walls being difficult to define. At about 24 hours, formation of the epicuticle begins in the head region and then progresses posteriorly. Paraffin sections of the body region show that most of the hypodermal cells have become elongated (20μ or more in length) as viewed in transverse section. In surface view they are approximately rectangular with measurements varying from 4–7μ by 7–12μ.

The newly formed epicuticle forms a very fine continuous membrane over the hypodermis and at this stage is not clearly separate from the overlying 4th instar cuticle. Well-defined epicuticular tubercles appear as needle-like

points on the dorsal integument and as indented cones ventrally. About 30–50 overlie each hypodermal cell, though there is much variation according to position, the tubercles being larger and less numerous ventrally than dorsally.

At about 12 hours prior to ecdysis the new epicuticle, except at the muscle insertions, becomes separated from the overlying 4th instar cuticle and



TEXT-FIG. 3. The formation of the 5th instar lava cuticle of *Diataraxia*. Transverse sections of developing cuticle: A, about 36 hours before ecdysis. B, about 12 hours before ecdysis. C, immediately before ecdysis. D, about 2 days after ecdysis.

moulting fluid begins to digest the latter. At the same time formation of the new endocuticle begins and pore canals and pore canal contents become visible (Text-fig. 3*b*). The latter are in the form of filaments each of which originates in the neighbourhood of the nucleus of a hypodermal cell and passes out through a pore canal in the endocuticle to an epicuticular tubercle. In Golgi preparations of the developing cuticle the pore canal contents are visible within the hypodermal cells either as solid staining filaments or as rows of minute dots.

When about 3–4μ of endocuticle has been laid down, the epicuticle and pore canal filaments begin to show strong basic staining properties. Groups of 5–12 pore canal filaments converge in the endocuticle to form a single main strand which enters the hypodermal cell. Thus each group forms a tree-like structure—a system characteristic of lepidopterous larvae (Plotnikow, 1904; Kuwana, 1933; Kühn and Piepho, 1938).

Sections of the soft cuticle of the moulting 4th instar larva just prior to ecdysis show that the old cuticle has been completely digested and that an approximately 10μ thick 5th instar outer endocuticle has been formed (Text-fig. 3*c*).

For some hours after ecdysis the caterpillar remains quiescent. During this period pigments are laid down in the hypodermal cells and epicuticle, and other processes take place which will be discussed later.

The pore canal filaments remain in contact with the hypodermis for 12–36 hours after ecdysis. Afterwards the formation of inner endocuticle, in which pore canals are absent, cuts them off in the outer endocuticle, where they remain as strongly basic staining filaments (Text-fig. 3*d*).

Measurements of hand sections of living cuticle showed that 5–6 days after moulting the inner endocuticle reaches a total thickness of about 50μ, of which about 30μ is laid down in the first $2\frac{1}{2}$ days. From $5\frac{1}{2}$ days until formation of the prepupa at about 7 days, little or no increase in thickness of the cuticle occurs. In Carnoy-fixed preparations of the developing larval cuticle of *Bombyx mori*, Kuwana (1933) observed an increase in thickness at the beginning and end of the instar with an intervening relatively quiescent period. If such measurements on fixed material are correct the mode of growth of the *Bombyx* cuticle is somewhat different from that of *Diataraxia*.

Formation of the Cuticulin Layer and Exocuticle

The young 5th instar epicuticle cannot be separated from the overlying 4th instar cuticle until 12–24 hours before moulting. A small portion was then removed and placed in chlorinated nitric acid in the cold. The attached hypodermis and newly formed endocuticle rapidly dissolved, leaving a fine transparent membrane which on heating broke down with the liberation of oily droplets. Thus as in *Rhodnius* (Wigglesworth, 1947) the cuticulin layer is well defined at an early stage. At this stage of development the staining reactions of transverse sections of the new cuticle are of interest. After Bouin fixation the newly formed cuticulin layer stains with eosin but not with iron haematoxylin, and in this respect has the same properties as the outer epicuticle of *Sarcophaga* (Dennell, 1946), but about 6 hours prior to moulting, when 4–5μ of endocuticle has been laid down, Bouin fixed, iron haematoxylin stained preparations begin to show deeply stained pore canal strands which appear to be discharging deeply staining material under the cuticulin layer to form the exocuticle. It should be noted that when this takes place, endocuticle formation has already begun, and thus the basic-staining material is laid down in the outer region of the endocuticle. This provides further evidence that the resulting layer contains chitin and is a true exocuticle.

It will be remembered that if the mature larval cuticle is treated with cold concentrated nitric acid the exocuticle is only dissolved after several hours. Now when a portion of the cuticle containing newly formed exocuticle is placed in cold concentrated nitric acid all layers except the cuticulin layer are rapidly destroyed. Thus the exocuticle is not yet in the mature resistant state nor does it show increase in resistance until after ecdysis.

Hardening of the Larval Exocuticle

In the development of typical hard cuticle, hardening and increase in resistance of the exocuticle are caused by tanning of exocuticular protein by quinones (Pryor, 1940 *a* and *b*). This process occurs at the same stage of development as the increase in resistance of the *Diataraxia* larval exocuticle, and the general similarity suggested that dihydroxyphenols are responsible for tanning in the thin exocuticle of soft cuticle.

The secretion of dihydroxyphenols in the developing cuticle may be studied by means of the argentaffin reaction (Lison, 1936), though it should be made clear that this test is not specific. However, material in the *Diataraxia* cuticle which colours red or red-black with ammoniacal silver hydroxide has been called dihydroxyphenol. Additional evidence to show that this conclusion is justified will be given later.

The old 4th instar cuticle of the moulting *Diataraxia* larva cannot be separated from the underlying 5th instar cuticle until 12–24 hours before moulting. If a portion of 5th instar cuticle is exposed at this stage and the larva immersed in 5 per cent. ammoniacal silver hydroxide for $\frac{1}{2}$ hour, washed, fixed in Carnoy, and the cuticle examined in surface view under the high power of the light microscope, it is seen that the new cuticle is only slightly stained. However, silver hydroxide has penetrated everywhere and heavily stained the underlying hypodermal cells. Prior treatment with cold chloroform for 5 minutes causes no significant difference in intensity or distribution of silver staining. Thus dihydroxyphenols are not present in the cuticle at this stage of development.

Some 6 hours before moulting the old cuticle can be separated at the muscle insertions and immersion in ammoniacal silver hydroxide as before again causes heavy staining in the hypodermis, showing that the cuticle is still permeable. The developing exocuticle stains light red, but at the muscle insertions it stains dark brick-red or black, indicating the presence of dihydroxyphenols. It was not possible to determine whether the cuticulin layer as distinct from the exocuticle caused reduction of ammoniacal silver hydroxide. However, after immersion in chloroform followed by treatment with ammoniacal silver hydroxide, many areas of the soft cuticle show brick-red staining droplets at the openings of the pore canals (Pl. II, fig. 14). This suggests that dihydroxyphenols are now being transported in the pore canals and that chloroform disrupts a layer which allows them to escape through the external openings of the pore canals.

It is remarkable that at this stage of development (about 6 hours before ecdysis) the cuticle is highly permeable to ammoniacal silver hydroxide and yet chloroform treatment is required to expose dihydroxyphenols in the pore canals. Moreover, the following evidence suggests that the pore canals themselves provide the path for penetration of ammoniacal silver hydroxide solution to the hypodermis: (1) electron micrographs show that the outer epicuticle which is hydrofuge does not extend over the openings of the pore canals; (2) between the wall of the pore canal and the pore canal strand is a well-defined annular space which prior to moulting provides a direct path through the endocuticle to the hypodermis; (3) after immersion of a larva with exposed new cuticle in ammoniacal silver hydroxide without prior chloroform treatment, particles are sometimes precipitated in the lumen of the pore canals but not within the body of the endocuticle.

Thus, it would appear that the lumen of the pore canals is not responsible for transport of dihydroxyphenols and it can only be assumed that these are transported by the pore canal strands which are themselves surrounded by some form of waterproofing film. If the latter is destroyed by chloroform treatment the dihydroxyphenols are released at the openings of the pore canals when they will then reduce ammoniacal silver hydroxide.

At about $\frac{1}{2}$ to 1 hour prior to moulting, permeability of the cuticle to ammoniacal silver hydroxide becomes reduced. The hypodermis stains less strongly and in some areas not at all. The exocuticle is also unstained, though the muscle insertions through which fibrils still pass to the old cuticle stain heavily. Dotted over the dorsal surface of the epicuticle are circular areas sometimes 10–15μ in diameter which stain intensely, but if the cuticle is first immersed in chloroform there is no sign of their presence. The significance of these areas is not understood.

If a larva at ecdysis is immersed in ammoniacal silver hydroxide for $\frac{1}{2}$ hour there is complete absence of staining everywhere. Thus, the waterproofing layer in *Diataraxia* is laid down just prior to ecdysis, an observation in agreement with those of Wigglesworth on *Rhodnius* (1947) and *Tenebrio* (1948), where, except over small areas, waterproofing is completed immediately before ecdysis.

For 1–2 days after ecdysis removal of the waterproofing layer with chloroform or by abrasion exposes the silver staining polyphenol droplets at the tips of the pore canals (Pl. II, figs. 15, 16). In older larvae they may sometimes be observed in limited areas generally around the limb bases, but elsewhere treatment with cold or hot chloroform fails to expose them, though light staining of the exocuticle and sometimes of the underlying hypodermis shows that ammoniacal silver hydroxide has penetrated the cuticle.

Harsh abrasion of the mature cuticle damages the body of the epicuticle and allows penetration of ammoniacal silver hydroxide which readily stains the abraded areas. Under the low power of the microscope the stained areas appear similar to those obtained after abrasion of the newly emerged larva, but, if such cuticle is examined in transverse section, it is clear that staining is not

due to droplets at the tips of the pore canals but is caused by ammoniacal silver hydroxide which has precipitated beneath the exocuticle.

To summarize: at about 6 hours before moulting, material probably containing dihydroxyphenol appears in the pore canals and continues to be secreted until 1–2 days after moulting. It is not discharged on to the outer surface of the epicuticle to form a layer. Therefore, the polyphenol layer as found in hard-bodied insects (Wigglesworth, 1947, 1948) is absent, perhaps a necessary precaution because excess dihydroxyphenol might induce further tanning in a cuticle which must retain its flexibility. The dihydroxyphenol is presumably secreted through the fine-branched filaments of the pore canal strands into the exocuticle and is responsible for limited tanning of this layer which as a result shows increased resistance to concentrated acids and alkalis. At about $1\frac{1}{2}$ days after moulting the hardening process ceases in the exocuticle. It is significant that at this stage the pore canals become cut off from the hypodermis and thus are unable to conduct and secrete further dihydroxyphenols.

Formation of the Cement Layer

As mentioned before, the cement layer can be readily stripped from the mature cuticle on a layer of collodion, deposited on a slide, and stained with iron haematoxylin. To determine when it is formed, this process was carried out on portions of cuticle at various stages of development. Examination showed that the cement layer was absent in all preparations obtained from the new cuticle prior to moulting, though in some an irregular net-like structure was stripped from the inter-segmental regions. This was considered to be an artifact produced by drying out of the digested remains of the old cuticle. Such artifacts could be produced artificially, but they were readily distinguishable under the electron microscope and never formed a continuous well-defined layer.

Collodion stripping of the cuticle at moulting and afterwards showed that a well defined cement layer had been formed. Thus as in *Rhodnius* and *Tenebrio* (Wigglesworth, 1947, 1948) the cement layer is laid down shortly before or at ecdysis.

Secretion of the Cement Layer

Wigglesworth (1947, 1948) has shown that in *Rhodnius* and *Tenebrio* the time of formation of the cement layer coincides with discharge of the dermal glands which he suggests are responsible for the formation of this layer. These observations suggested a study of the dermal glands of *Diataraxia*.

In lepidopterous larvae the large segmentally arranged glands were first described by Verson (1890). A pair in each segment lie laterally just above the limb bases in the thorax and in a similar position in the 8th abdominal segment. In the thoracic segments and in the 1st–8th abdominal segments a gland is situated dorso-laterally above and just anterior to each spiracle. Each gland consists of three modified hypodermal cells: the canal cell, the intercalary cell, and the large gland cell.

Development of Verson's Gland in the 4th–5th Instar of the Diataraxia *larva*

In *Diataraxia* the large gland cells are either barrel-shaped (ventro-lateral thoracic glands) or flattened and somewhat heart-shaped (dorso-lateral glands; Pl. II, fig. 17). First, their development was studied in detail by examination of 10μ sections of moulting 4th–5th instar larvae which were stained in Mallory or iron haematoxylin and eosin. Bouin and Carnoy were used as fixatives. These preparations confirmed the observations of Schürfeld (1935) regarding the period of development and time of discharge of the glands. At cessation of feeding prior to beginning of the moulting period the glands show an ill-defined basic staining central nucleus and an acidic staining cytoplasm containing numerous small vesicles (Pl. II, fig. 18). During the next 24 hours the glands and the vesicles enlarge somewhat and the nucleus begins to show a well-defined branched structure. At the end of this period, about 24 hours before ecdysis, preparations show that each vesicle is surrounded by a well-defined membrane and contains one or more granules or droplets of material with basic staining properties similar to the nucleus (Pl. II, fig. 19; Pl. III, fig. 20). The granules, which were observed by early investigators but not mentioned in more recent publications, are sometimes relatively large and in the form of droplets in ventro-lateral thoracic glands but are smaller in the dorso-lateral glands (Pl. III, fig. 21). Mallory-stained sections show that the droplets are not homogeneous but contain lightly stained particles (Pl. III, fig. 20). Noticeable development of the gland occurs in the 12 hours prior to ecdysis. The large gland cell increases in size and the cytoplasmic vesicles enlarge and apparently coalesce to some extent. The basic staining droplets apparently divide into smaller particles and finally break down completely, so that each vesicle becomes filled with a thinly dispersed material (Pl. III, fig. 22). Immediately prior to discharge the walls of the vesicles disintegrate and the nucleus is pushed to one side of the single lumen which is now filled with secretion. It would thus appear that solid material is actively secreted into the gland vesicles and that the secretion is not merely a watery fluid as suggested by Schürfeld (1935) and Kühn and Piepho (1938).

Further experiments were carried out to determine the nature of the large gland cell secretion. Individual glands were dissected in Ringer's solution from larvae at various stages of development. On treatment with 1 per cent. α-naphthol in 95 per cent. alcohol followed by concentrated sulphuric acid (Molisch's test), the response was negative. This suggested the absence of glucoproteins which as mucin might be expected to act as a lubricant facilitating the casting of the old cuticle. Response to the nadi reagent (Lison, 1936) was also negative. This suggested the absence of the oxidase systems and of phenolic materials (Dennell, 1947). Prolonged treatment in the nadi reagent (10–20 minutes) showed pale staining granules in some glands. These were also observed in unfixed glands treated with 0·05 per cent. methylene blue in Ringer's solution. Clearly the basic staining granules observed in sectioned material are present in the living cell and are not an artifact of fixation as sug-

gested by Verson (1911) and Schulze (1912). Plotnikow (1904) thought that they were chromatic droplets, and to test this suggestion a series of glands were slightly macerated on coverslips, fixed in saturated mercuric chloride + 2 per cent. acetic acid, and then subjected to the Feulgen reaction (Lison, 1936). The granules, however, did not stain. It is of interest to record the distribution of nucleoprotein as shown by these preparations. In the actively secreting cell about 12 hours prior to ecdysis the nucleus ramifies throughout the cell and nucleoprotein in the form of well-defined minute particles is present over much of the wall of each vesicle. When secretion ceases and the vesicles break down, the nucleoprotein becomes aggregated and fibrous in appearance. Clearly the nucleus is actively concerned in secretion, but the material secreted into the vesicles does not contain desoxyribonucleic acid.

To test for protein the ninhydrin test was carried out by warming with ninhydrin in 50 per cent. glycerol (Wigglesworth, 1942). With unfixed glands gentle warming produced an immediate positive response and small droplets in the gland vesicles showed strong purple staining. Prior fixation with Bouin or 10 per cent. formalin resulted in much reduced response to ninhydrin. The nucleus became coloured, but elsewhere only a diffuse and pale-blue coloration was obtained.

To test for lipoid, glands attached to coverslips as before were fixed in Baker's formaldehyde and stained in sudan black B (Pantin, 1948). Preparations showed that each vesicle contained a large staining droplet (Pl. III, fig. 23). The droplets are well defined by about 24 hours before ecdysis when they stain rather lightly, though the surrounding protoplasm stains strongly even after immersion in the dye for periods as short as 3 minutes. In later stages of development the droplets begin to stain more heavily, and as the vesicles enlarge they enlarge correspondingly and throughout development probably fill each vesicle, though in unfixed preparations they appear to have shrunk. Just before ecdysis when the vesicle walls break down, the droplets coalesce. Thus Verson's gland secretion contains a lipoid. Probably it is the presence of this lipoid which after fixation in formaldehyde masks the response of the protein to ninhydrin. Moreover, if a gland is fixed in formaldehyde and treated with iron haematoxylin the contents of the vesicles fail to stain (Pl. II, fig. 17) unless the lipoid is first removed with alcohol. Glands fixed in Baker's formaldehyde were transferred to absolute alcohol overnight and several were also fixed in Carnoy which would undoubtedly remove any lipoid present. They were then immersed in sudan black B and examined. The large sudan-staining droplets had disappeared and the gland vesicles were empty except for the granules or thinly dispersed material similar to those visible in the haematoxylin-stained sections.

Thus, it is concluded that lipoid as well as protein is secreted by Verson's gland. The relationship of the haematoxylin- and Mallory-staining protein granules to the sudan-staining droplets cannot be determined with certainty from fixed preparations. Mallory-stained paraffin sections of the ventral thoracic glands 24 hours before ecdysis show droplets which resemble the

sudan-staining droplets, but at later stages of development these droplets or granules occupy but a small part of the vesicle, while the sudan-staining droplets probably fill it completely. Moreover, the lipoid can be removed from the vesicles with absolute alcohol, leaving the granules undissolved. This suggests that protein granules are present as distinct particles within each sudan-staining droplet. However, just before ecdysis the granules break down into a thinly dispersed material and thus it seems probable that the secretion at discharge consists of an emulsion containing protein which is now associated with the lipoid in the form of a lipoprotein.

It is interesting that glands fixed in 2 per cent. osmium tetroxide for 10 minutes turn but light brown in colour. If the gland is slightly macerated, refractile droplets, which are not miscible with Ringer or osmic solution, escape and become clearly defined after osmic treatment, but show little or no staining response (Pl. III, fig. 24). No further staining occurs even after immersion in osmium tetroxide for 24 hours. Now it has been shown that many lipoprotein complexes in cells and body fluids fail to respond to various tests for lipoids, although it can be demonstrated that considerable quantities of saturated and unsaturated lipoids are present (Lovern, 1942). These complexes are dissociated and the lipoid removed by treatment with absolute alcohol. This information may explain the absence of response to stains of the type B dermal gland of *Rhodnius* (Wigglesworth, 1947, 1948). The glands contain a large distended oval vesicle whose contents colour pale violet with ninhydrin but do not stain with iron haematoxylin, osmium tetroxide, or other fat stains. There seems little doubt that these glands secrete a lipoprotein. Perhaps this is also true of dermal glands in the tick *Ornithodorus*, where the gland lumen becomes filled with a 'colourless secretion' (Lees, 1947).

Discharge of Verson's Gland

The moulting larva remains quiescent until about 10 minutes before ecdysis, when muscular contractions begin throughout the body. These consist of dimpling movements of the cuticle and some waves of contraction. The muscle insertions on the old epicuticle are detached during this process, and the limbs and tracheal exuviae partly withdrawn. At the same time, as observed by Schürfeld (1935), a liquid is discharged into the space between the old and the new cuticle. To determine whether this liquid was discharged by Verson's glands a moulting larva of *Diataraxia* was fixed with Bouin, just as liquid secretion was beginning. Sections showed that several of the glands were in the act of discharging a thinly dispersed material which was visible in the exuvial space. A gradient in time of discharge was noticed—anterior glands having discharged their secretion whilst posteriorly the glands were still undischarged. In addition, the act of discharge was observed by removing the old cuticle from the abdomen of a moulting *Diataraxia* larva just as the process was taking place. Liquid was emerging exactly where the gland duct opened in a position dorsal and just anterior to the spiracles. The liquid, which appeared transparent, ran along the cuticle folds and in a few moments dried up. The

larva was then immersed in 5 per cent. ammoniacal silver hydroxide for ½ hour. The gland secretion was defined by faint red-silver staining.

Relation of Verson's Gland Secretion to Formation of the Waterproofing Layer

The old cuticle was removed from the abdomen of a moulting 4th instar larva just as Verson's glands were about to discharge, and the exposed new cuticle was immersed in ammoniacal silver hydroxide for ½ hour. Silver staining of the cuticle was limited to some of the muscle insertions and to a number of the previously mentioned areas, the significance of which is not understood. Thus, before Verson's glands have discharged, waterproofing is practically complete. This suggests that Verson's glands are not responsible for secretion of the waterproofing layer. Confirmation was obtained by removing the exuvial head capsule of a larva some hours prior to moulting when the new cuticle was still permeable to water and stained readily in ammoniacal silver hydroxide. Celloidin enamel was used to glue the cut edge of the body exuvium to the new cuticle just behind the head. This prevented passage of secretion from the exuvial space on to the larval head which itself contains no dermal glands. When Verson's gland had secreted and the larva was attempting to moult, the head was immersed in ammoniacal silver hydroxide for 1 hour. No staining occurred, thus showing that the waterproofing wax layer had been formed in the absence of Verson's gland secretion.

Verson's Glands and the Formation of a Cement Layer

In the moulting *Diataraxia* larva it has been shown that discharge of Verson's glands and formation of a cement layer both occur just before the end of ecdysis. Now these two processes must occur almost simultaneously. Cement layer formation over the muscle insertions cannot take place until the muscle-fibre attachments between the old and new cuticle have been broken. The latter process occurs about 10 minutes prior to ecdysis and thus the cement layer must be formed during the following 10 minutes. During this period no observed secretory process takes place except the discharge of Verson's glands, and thus there seems little doubt that as in *Rhodnius* and *Tenebrio* (Wigglesworth, 1947, 1948) the dermal glands are responsible for secretion of the cement layer. The nature of the gland secretion suggests that the cement layer consists of a lipoprotein.

Changes following Discharge of Verson's Gland

Directly after secretion, the large gland cell collapses and preparations show a rather ill-defined nucleus and disorganized cytoplasm. However, in some glands, vesicle formation begins in the cytoplasm just after ecdysis, though little further change occurs until the beginning of the next moult.

Previous investigators have noticed that just after the large gland cell has discharged, the exit duct is blocked by a plug of material, well defined by its ability to stain intensely with haematoxylin. Schürfeld (1935) suggested that this consisted of swollen fragments of the lining of the gland duct which

had been partially digested by a secretion from the intercalary and canal cells. However, in *Diataraxia* the duct lining of the canal cell appears to remain intact and may be observed surrounding the plug. The lining of the duct in the intercalary cell is extremely thin and generally ill defined and it is difficult to conceive how in *Diataraxia* a relatively large plug is formed by digested fragments of such a duct wall.

The following observations provide information on the structure and formation of the plug. The plug is dark brown in colour. This indicates the presence of phenolic materials and suggests that it consists of tanned protein. Now the intercalary cell stains strongly with ammoniacal silver hydroxide and moreover gives a positive nadi response in the presence of cyanide (Dennell, 1947). This suggests that the intercalary cell secretes a dihydroxyphenol which would form the necessary quinone for the tanning process. The source of protein is not known. Perhaps in addition, the intercalary cell discharges dihydroxyphenol into the large gland cell secretion as it passes through the gland duct. This would account for the ability of the discharged secretion and of the cement layer to stain with ammoniacal silver hydroxide and would suggest that the cement layer consists of a lightly tanned lipoprotein.

Comparison of Hard and Soft Cuticle

It has been shown already that limited hardening occurs in the exocuticle of soft cuticle and that the material responsible is probably a dihydroxyphenol transported from the hypodermis by means of the pore canals. However, certain small areas of the *Diataraxia* cuticle consist of typical hard cuticle with a well-defined exocuticle and pore canal system essentially similar to that of hard-bodied insects (Wigglesworth, 1933, 1948; Richards and Anderson, 1942). In these insects the pore canals are connected to the hypodermis throughout the life of the insect, a condition which appears to be fundamentally different from that of soft cuticle.

Properties of Hardened Cuticle of Diataraxia

In the hardened areas the cuticulin layer is over $0{\cdot}5\mu$ thick, compared with about $0{\cdot}2\mu$ for soft cuticle, and it has a flat as distinct from tuberculate surface. 2μ Mallory-stained transverse sections of the mature hardened cuticle show the pore canals as fine red-staining strands passing from the hypodermis through the inner endocuticle, branching on the exocuticle, and then penetrating the cuticulin layer (Pl. III, fig. 25). Both the pore canals and the strands they contain are finer and less readily distinguishable than those of soft cuticle. However, penetration of the pore canals through the cuticulin layer to the surface or to an unmeasurable distance beneath it is very distinct, thus agreeing with the observations of Wigglesworth (1948), but differing from those of Richards and Anderson (1942) who state that in *Periplaneta* the pore canals end blindly in the endocuticle.

The exocuticle generally stains strongly with iron haematoxylin, though in the hardest areas the staining ability is lost. It also stains in ammoniacal silver

hydroxide, suggesting that phenolic materials are present and that the method of hardening is the same as in typical hard-bodied insects, namely, the tanning of cuticular protein by quinones (Pryor, 1940 *a* and *b*). If the complete larval cuticle of *Diataraxia* is cleaned from body tissues and examined in surface view and transverse section after immersion overnight in ammoniacal silver hydroxide, the exocuticle of soft cuticle is stained, though not strongly, but the hardened areas form well-defined black or brick-red staining patches of exocuticle, the staining being heaviest in the mouth parts, proleg crotchets, head, and fore limbs, weaker in prothoracic and last abdominal tergal shields, and weakest in small shield-shaped pads on the outer surface of the prolegs. This ability to reduce ammoniacal silver hydroxide is related to degree of hardness and, moreover, the natural colour varies from light amber in the hardest to colourless in the least hardened areas.

Table VI shows the properties of these areas compared with those of (1) hardened cuticle at moulting, and (2) typical soft cuticle.

TABLE VI. *Properties of Hard and Soft Cuticle of the* Diataraxia *Larva*

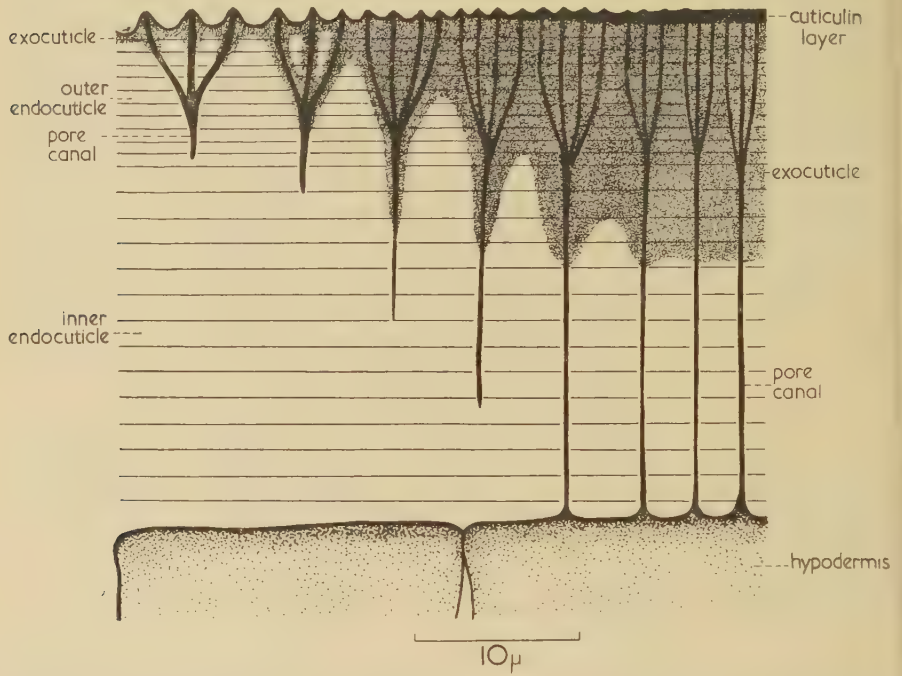
	Hard areas of cuticle			*Soft cuticle*
	Mature exocuticle			
	Hardest areas	*Least hardened areas*	*Exocuticle at moulting*	*Mature exocuticle*
Natural colour	Light amber	Colourless	Colourless	Colourless
Response to ammoniacal silver hydroxide	Stains black or brick-red	Stains brick-red	Stains brick-red	Stains light red
Response to iron haematoxylin	Staining variable, sometimes slight or not at all	Stains heavily	Stains heavily	Stains heavily

It is clear that the above series represent intermediate stages in the development of typical hard cuticle (cf. Pryor, 1940*b*). On this basis it would appear that typical phenolic tanning occurs in the soft cuticle as well as in the hard cuticle of the *Diataraxia* larva, the only notable difference being the extent of tanning, which in the former occurs to a limited extent in an exocuticle 0·5 μ thick, but in the latter occurs extensively in an exocuticle which may be over 11 μ thick.

Secretion of Dihydroxyphenol and Protein

Kühn and Piepho (1938) showed that in partial pupation spots of *Ephestia* strongly basic-staining material is secreted through the developing epicuticle and may form droplets in the cavity between the old and the new cuticle. Partial pupation was similarly induced in the pupating *Diataraxia* larva. The droplets not only stained with haematoxylin but gave a positive ninhydrin response after fixation in 10 per cent. formaldehyde and also stained red in ammoniacal silver hydroxide. This suggests that they contain protein and

dihydroxyphenol. The discharge of this material occurs at the same period as the discharge of the material containing dihydroxyphenol observed by Wigglesworth in *Rhodnius* (1947) and *Tenebrio* (1948), and there seems little doubt that the two secretions are homologous. Wigglesworth (1947, 1948) showed that the pore canals were responsible for secretion of 'material' containing dihydroxyphenol, and thus the present findings provide evidence to substantiate his



TEXT-FIG. 4. Transverse section through the edge of a proleg pad showing transformation from soft to hard cuticle. Transformation occurs gradually over a length of about 200 μ, but in this diagram the various stages have been compressed into about 40 μ.

suggestion that the material consists of protein and that both protein and dihydroxyphenol are secreted by the pore canals. Moreover, a study of the slightly hardened areas of the *Diataraxia* cuticle provides additional evidence. Transverse sections of a proleg pad show gradation of structure from the condition typical of soft cuticle on the outside to that of hard cuticle towards the centre of the pad. The gradation is characterized by an increase in thickness of the cuticulin layer, flattening of the tubercles and extensive development of the exocuticle (Text-fig. 4). At the edge of each pad the pore canals are typical of soft cuticle (Pl. I, fig. 8) and are confined to the outer endocuticle, but towards the centre they extend deeper into the inner endocuticle until finally they connect with the hypodermis. The distribution of basic-staining exocuticular protein in relation to the pore canals is important. Transverse sections show that at the edge of the pad of hardened cuticle each pore canal is

surrounded by a narrow basic-stained area of exocuticle. Increase of the staining area round each pore canal (Pl. III, fig. 26), followed by coalescence to form a thick exocuticle, coincides with the extension of the pore canals into the inner endocuticle (Text-fig. 4). Thus, the longer the period during which the pore canals are in contact with the hypodermis the greater is the extent of secretion of exocuticular protein. This provides conclusive evidence that the protein is transported by the pore canals.

Preparations of developing cuticle of the *Diataraxia* larva show that discharge of protein and dihydroxyphenol by the pore canals begins about 6 hours before ecdysis. In soft cuticle these materials form a thin exocuticle, the process being complete by about 1–2 days after ecdysis. In the hard areas of cuticle the process of exocuticle formation is much more extensive. Secretion of exocuticle material begins in the outermost layers of the endocuticle and then extends inwards. Protein deposition keeps ahead of tanning as shown by the fact that in the *Diataraxia* cuticle the inner limit of the exocuticle as defined by its ability to stain with iron haematoxylin is greater than the limit of the area which reduces ammoniacal silver hydroxide. In the *Diataraxia* cuticle the process of hardening in such areas as the head, although occurring mainly during the first day after moulting, continues to a limited degree during the later development of the instar. Thus, 1–2 days after moulting the thickness of the exocuticle (the silver-reducing area) in the head capsule is about 7μ. On the fifth day it has generally increased to about 11μ and in some areas an inner and less heavily stained area of exocuticle about 8μ thick has also been formed. This suggests that hardening materials continue to be transported from the hypodermis to the exocuticle for some time after ecdysis, and helps to explain why the pore canals of hard cuticle maintain continuous contact with the hypodermis.

Functions of the Pore Canals

The functions of the pore canals have been discussed previously by a number of authors and it is generally agreed that they act as a conducting system between the hypodermis and the outer layers of the cuticle. Wigglesworth (1947, 1948) has shown that the pore canals are responsible for conduction and secretion of dihydroxyphenols in the developing cuticle of *Rhodnius* and *Tenebrio*, though it is of interest that in *Sarcophaga*, where hardening occurs in the mature larval cuticle, dihydroxyphenols are transported in the absence of pore canals (Dennell, 1947). There is no evidence to support suggestions that the pore canals are responsible for transport of enzymes, proteins, and waxes, while Dennell (1946) has shown that secretion of endocuticular material by the pore canals is unlikely.

The present findings, based on the studies of the developing soft and hard cuticle of the *Diataraxia* larva, have given some information on the functions of the pore canals. The conclusions may be summarized as follows: in the cuticle two forms of conducting pore canal system may be distinguished. The first in soft cuticle functions only during the early stages of development and

the second in hard cuticle is functional for a much longer period, probably throughout the life of the instar. In soft cuticle the pore canal strands transport basic-staining protein to form the exocuticle and also dihydroxyphenols which presumably are oxidized to quinones and cause limited tanning of the exocuticular protein. With regard to *Diataraxia* it is of interest that when the limited tanning of the exocuticle has been completed the hardening process is extended to the pore canal strands in which the residual protein and phenol apparently react as in the exocuticle. The pore canals then become cut off from the hypodermis by the developing inner endocuticle.

In hard cuticle protein and dihydroxyphenol secretion by the pore canals is more extensive and the degree of tanning of the exocuticle more nearly complete. This process, which continues for a much longer period than in soft cuticle (perhaps throughout the life of the insect or instar), requires the maintenance of a conducting pore canal system from the hypodermis.

Discharge of the Moulting Fluid

The hypodermal cells are responsible for secretion and afterwards for reabsorption of the moulting fluid (Wigglesworth, 1933). During both these processes the moulting fluid has to pass through the developing new cuticle. Now, in the moulting *Diataraxia* larva it has been shown already that prior to ecdysis the pore canal lumen provides a direct path for conduction of water-soluble materials, such as ammoniacal silver hydroxide, through the cuticle from the exuvial space to the hypodermis. In particular the external openings of the pore canals would appear to provide the only path whereby water-soluble materials can pass through the hydrofuge cuticulin layer. Thus it is suggested that the pore canal lumen is responsible for transport of the moulting fluid.

Transport of Waterproofing Waxes through the Cuticle

If pore canals are required for the transport of waterproofing waxes, the soft cuticle of *Diataraxia* larva should be incapable of recovery of waterproofing after abrasion because the 50μ thick inner endocuticle is devoid of pore canals. Therefore it is of interest to study the phenomenon of recovery of waterproofing in this insect.

With *Diataraxia* it was found that serious errors were involved in studying water loss over a period of days with the same individual. In the first place the larva is an active feeding stage, and determination of weight loss over a period of hours is complicated by the ejection of faeces. This was prevented by starving the larva, blocking the anus, and inactivating it by ligaturing the head. However, the rate of water loss of such an abraded larva in dry air, exposed only for short periods, caused such depletion of body water that recordings of water loss on subsequent days showed an abnormally low figure. Thus a batch of five larvae of average weight of 0·28 gm. showed a water loss of 19·4 mg. per larva per hour during a 3-hour exposure to dry air directly after abrasion. They were kept at 100 per cent. relative humidity until

the next day, when they showed a water loss of 10·3 mg. per larva. On the third day the larvae had shrivelled: two were dead and the rate of loss was 3·2 mg. per larva. A separate batch similarly abraded and then kept continuously at 100 per cent. relative humidity showed a water loss of 19·2 mg. per larva per hour in dry air 4 days after abrasion. Thus, the apparent recovery of waterproofing in the first batch was caused entirely by depletion of body water. It should be noted that a decapitated larva seems incapable of recovery of waterproofing.

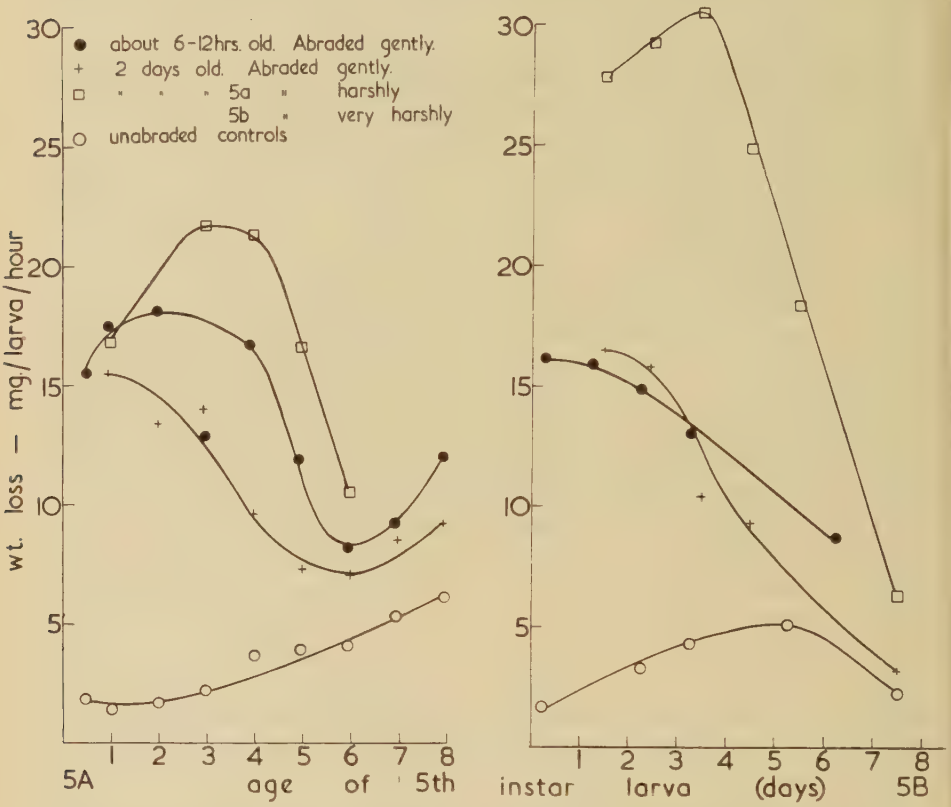
The following procedure was adopted. Batches of larvae were abraded by rubbing the dorsal surface of the first six abdominal segments with a piece of paper dipped in Almicide dust. As far as possible the procedure was standardized and an attempt was made to abrade a similar area of each larva. Adhering dust was washed from each larva and five larvae immediately removed, ligatured round the neck and killed in ammonia vapour. The ligature was carried out to prevent regurgitation which might contaminate the cuticle. Sources of water loss were then carefully occluded. Efficient occlusion of the mouth was not easy, and accordingly the head was removed and the cut end of the neck, the anus, and the spiracles covered with a spot of celloidin solution. The larvae were hung in a small desiccator over phosphorus pentoxide at 25° C. for 1 hour. They were then weighed, replaced in the desiccator and reweighed after a further 2 hours to determine water loss during this period, after which they were discarded. The remaining abraded larvae were placed in dishes with food and kept at 25° C. and 100 per cent. relative humidity. On the following day a further five larvae were removed and treated as above, this process being continued at daily intervals until the larvae were 8 days old.

Three batches of about forty larvae were abraded and a fourth control batch, apart from being unabraded, was treated in the same way as the others in every respect. Of the abraded larvae one batch consisted of 5th instar larvae abraded within about 12 hours after moulting. It will be remembered that in the cuticle of larvae of this age, functional pore canals still connect the hypodermis to the epicuticle. Abrasion of this batch was carried out by exerting slight pressure on the filter-paper, a process which removes the waterproofing layer and generally exposes silver-staining droplets at the tips of pore canals. Text-fig. 5*a* shows that water loss expressed as mg. per larva per hour increased somewhat during the 2 days following abrasion—it then decreased until it reached a figure which remained approximately constant at a level somewhat higher than that of the control.

The other two batches of treated larvae were abraded at about 2 days after moulting when the pore canal contents had become chitinized and sclerotized and a 10–20μ thick inner endocuticle without pore canals had been formed. At this stage silver-staining areas are seldom exposed when larvae are abraded gently. Rather more harsh abrasion, besides removing the waterproofing layer, knocks off the tips of the tubercles and allows ammoniacal silver hydroxide to penetrate the epicuticle and precipitate beneath it. One batch of larvae was abraded in this way whilst the other was abraded gently, as was the

first batch. Text-fig. 5*a* shows that rates of transpiration directly after abrasion were approximately the same in both treatments. On succeeding days it decreased steadily in the gently abraded batch, but the harshly abraded larvae showed an initial increase, though after 2–3 days there was a rapid decrease.

The above results were confirmed in a second experiment. Four batches of 5th instar larvae were used: (1) abraded gently at about 6 hours old; (2)



Text-fig. 5. Graphs showing recovery of waterproofing after the abrasion of the 5th instar cuticle of *Diataraxia*. Batches of larvae were abraded and transpiration in dry air at 25°C. was determined at intervals during the following seven days. Explanation in text.

abraded gently at about 2 days old; (3) abraded severely and extensively at about 2 days old; (4) unabraded controls. Text-fig. 5*b* shows the results. The first two treatments showed a gradual decrease in water loss, the second reaching a final level very similar to the control. The third treatment showed an initial increase and then a very rapid decrease.

There was a slight difference of procedure in this experiment. Prior to determination of water loss, the larvae were not killed with ammonia but were ligatured round the neck, the head removed, and only the neck and anus blocked with celloidin. The fact that they were alive may account for the

difference in water loss between living and dead larvae after about $5\frac{1}{2}$ days (cf. Text-fig. 5, *a* and *b*). At this stage larval contraction begins prior to prepupal formation. In the living larva contraction by decreasing surface area might cause a reduction of transpiration, whereas the dead individual would be relaxed, and decrease in transpiration at prepupal formation would not be apparent.

The data demonstrate that larvae are capable of recovery of waterproofing after abrasion and, moreover, they show that there is no significant difference between rates of recovery of larvae abraded when the pore canals connect with the hypodermis, and of those abraded when the pore canals have become cut off by the inner endocuticle and their contents sclerotized. This suggests that the pore canals are not responsible for secretion of waterproofing waxes.

The final levels of transpiration reached by abraded larvae—somewhat higher than that of the controls—show that waterproofing is generally not completely restored, a fact previously demonstrated by Wigglesworth (1945) and Lees (1947). However, in one gently abraded batch the difference, if significant, was slight.

The process of recovery of waterproofing requires consideration. Electron micrographs show that the tips of the tubercles may be removed by harsh abrasion. The initial rise of transpiration in harshly abraded larvae would seem to occur when such damage to the framework of the epicuticle is being repaired. Only after this process has been completed can the superficial waterproofing wax layer be laid down. In some larvae the regenerated wax was observed as a white bloom easily rubbed off the cuticle, which melted into oily droplets when warmed (cf. Wigglesworth, 1945).

Thus, during recovery after abrasion, and possibly also during formation of the waterproofing layer prior to ecdysis, waterproofing waxes can pass outwards through the substance of the endocuticle. It is possible that the waxes are discharged on to the epicuticle surface through the external openings of the pore canals, but as the epicuticle is readily permeable to lipoid materials (Wigglesworth 1945) there seems no doubt that waxes can pass more readily through the framework of this layer than through the pore canals which apparently have hydrophil properties.

Acknowledgements

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EXPLANATION OF PLATES

Plate I

Fig. 1. Electron micrograph of a 2μ thick transverse section of the dorsal cuticle showing outlines of the epicuticular tubercles. Note irregular shaped swellings at the tips of the tubercles. R.C.A. microscope. 50 kv. Magnification × 9,000.

Fig. 2. Electron micrograph of the surface of the cuticulin layer obtained by treatment of new cuticle just prior to ecdysis with cold concentrated hydrochloric acid overnight. The tubercle has collapsed on one side owing to removal of the supporting exocuticle. Nickel shadowed. R.C.A. microscope. 50 kv. Magnification × 23,600.

Fig. 3. Photograph of the cement layer in surface view showing the layer where it overlies part of two muscle insertions. Stripped with collodion. Overstained with iron haematoxylin. Magnification × 450.

Fig. 4. Electron micrograph of the cement layer in surface view. Siemen's microscope. 50 kv. Magnification × 5,200.

Fig. 5. Electron micrograph of the cement layer at the edge of a muscle insertion. The tubercles are completely covered by the layer. Note plugs of material removed from the openings of the pore canals. The fine granular particles are probably impurities from the collodion used in the stripping process. Siemen's microscope. 50 kv. Magnification × 10,700.

Fig. 6. Electron micrograph of the cement layer overlying the dorsal epicuticle of the exuvium. To differentiate the cement layer, the exocuticle was partly removed with concentrated hydrochloric acid. Presumably the exocuticle is still present in the tubercles which appear opaque. Siemen's microscope. 100 kv. Magnification × 5,300.

Fig. 7. Electron micrograph of the lateral epicuticle in surface view. The exuvium was treated overnight with 5 per cent. pepsin (pH 2, 32° C.), which removes the plugs blocking the openings of the pore canals at the tips of the tubercles. R.C.A. microscope. 50 kv. Magnification × 15,600.

Fig. 8. Photograph of a transverse section of the mature lateral cuticle. In the outer endocuticle the tree-like pore canal strands stain strongly. One strand enters each epicuticular tubercle. Stained with iron haematoxylin. Magnification × 825.

Fig. 9. Electron micrograph of lateral epicuticle in surface view. The cuticle was treated with concentrated hydrochloric acid for 10 minutes to remove the endocuticle. The pore canal strands are unaffected by this treatment and remain attached to the undersurface of the epicuticular tubercles. Siemen's microscope. 100 kv. Magnification × 9,200.

Fig. 10. Electron micrograph of an epicuticular tubercle in surface view. Stained with Altmann to define the fine filaments which connect each pore canal strand to the epicuticle or exocuticle. Siemen's microscope. 100 kv. Magnification × 10,200.

Fig. 11. Photograph of a surface section of the outer endocuticle from the ventral integument between the prolegs. Fixed with Flemming and stained with iron haematoxylin to show the pore canals and pore canal strands in transverse section. Magnification × 825.

Fig. 12. Electron micrograph of part of a lamella from the outer endocuticle treated 48 hours with 2 per cent. pepsin (pH 2, 32° C.) and examined in surface view. Pore canals and indications of chitin fibres are visible. R.C.A. microscope. 60 kv. Magnification × 12,600.

Plate II

Fig. 13. Electron micrograph of part of the same lamella as Pl. I, fig. 12, treated overnight with 5 per cent. potassium hydroxide at 60° C. and examined in surface view. Chitin fibres and bundles of fibres are well defined. Nickel shadowed. R.C.A. microscope. 50 kv. Magnification × 37,100.

Fig. 14. Photograph of the surface of the new epicuticle about 6 hours prior to ecdysis after immersion in cold chloroform for 5 minutes followed by 5 per cent. ammoniacal silver hydroxide for half an hour. The photograph is taken at a slightly oblique angle. 'Polyphenol' droplets may be seen on the tips of the tubercles at the openings of the pore canals. The cement layer is absent. Magnification × 825.

Fig. 15. Photograph of the surface of the epicuticle about 24 hours after ecdysis after treatment as described for fig. 14. 'Polyphenol' droplets are exposed at the openings of the pore canals and the cement layer shows faint silver staining. Magnification × 450.

Fig. 16. Photograph of the surface of the epicuticle about 24 hours after ecdysis, after abrasion with Almicide dust and immersion in 5 per cent. ammoniacal silver hydroxide for half an hour. In the abraded regions 'polyphenol' droplets are exposed at the tips of the pore canals. Magnification × 450.

Fig. 17. Photograph of a whole mount of a mature Verson's gland from the abdomen of a moulting 4th–5th instar larva. The canal cell is absent. The diffusely branched nucleus of the large gland cell is well defined. Fixed in Baker's formaldehyde and stained with iron haematoxylin and light green. Magnification × 50.

Fig. 18. Photograph of 10 μ section of the large gland cell of Verson's gland from a moulting 4th 5th instar larva about 2 days before ecdysis. Fixed in Bouin and stained with iron haematoxylin and eosin. Numerous small vesicles are present in the cytoplasm, but the nucleus is not well defined. Magnification × 225.

Fig. 19. Photograph of a 10 μ section of a 2nd ventral thoracic Verson's gland about 24 hours before ecdysis, treated as described for fig. 18. The basic staining contents of the vesicles and the branched nucleus of the large gland cell are well defined. The intercalary cell is also present. Magnification × 225.

Plate III

Fig. 20. Photograph of a 10 μ section of a large gland cell from the 3rd ventral thoracic segment at about the same stage as Pl. II, fig. 19. Fixed in Carnoy and stained with Mallory. Each

vesicle is surrounded by a membrane and contains an unevenly stained droplet. Branches of the nucleus ramify amongst the vesicles. Magnification × 975.

Fig. 21. Photograph of a 10 μ section of a large gland cell from the 4th abdominal segment at a rather later stage than Pl. II, figs. 19 and 20, treated as described for Pl. II, fig. 18. Each vesicle contains a number of small droplets or granules. Magnification × 225.

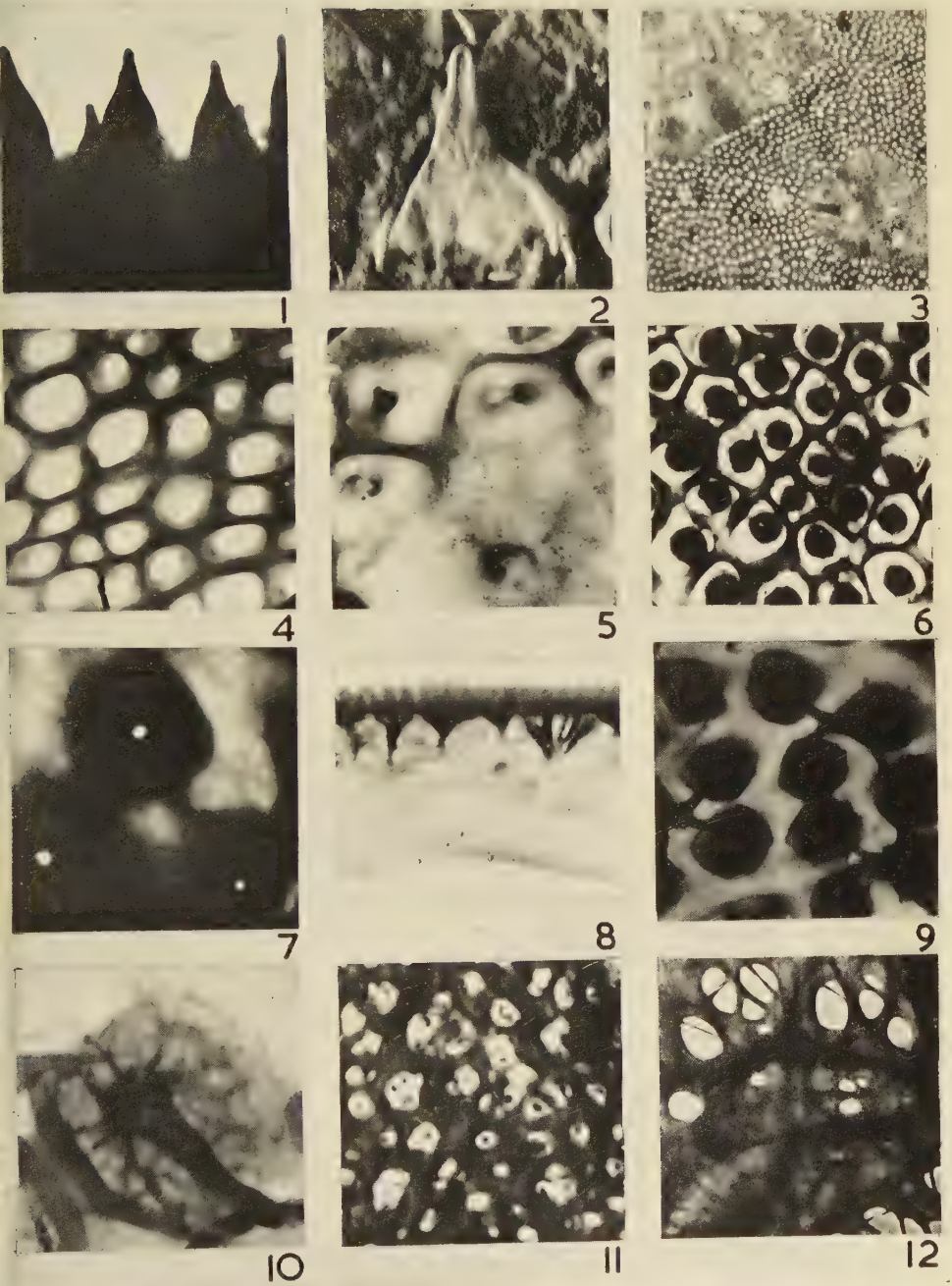
Fig. 22. Photograph of a 10 μ section of a large gland cell from the 3rd abdominal segment just prior to breakdown of the vesicle walls. Treated as described for Pl. II, fig. 18. The vesicle contents consist of a thinly dispersed material. Magnification × 225.

Fig. 23. Photograph of contents of a macerated abdominal gland at about the same stage of development as fig. 21. Fixed in Baker's formaldehyde and stained with sudan black B to demonstrate lipoid. Both vesicle contents and the surrounding protoplasm stain readily. Magnification × 450.

Fig. 24. Photograph of contents of a macerated abdominal gland from a larva at about the same stage of development as fig. 21. Fixed in 2 per cent. osmium tetroxide. Vesicles in the body of the gland are visible and from some of them the contents have escaped in the form of unstained refractile droplets. Magnification × 450.

Fig. 25. Photograph of a 2 μ transverse section through the hard cuticle of a thoracic leg of the 5th instar larva. The pore canals may be seen passing through both the endocuticle and the epicuticle and apparently opening on the surface. Fixed in Carnoy and stained with Mallory. Magnification × 825.

Fig. 26. Photograph of a 6 μ transverse section through the cuticle at the edge of a proleg pad. The pore canals are beginning to extend into the inner endocuticle and each is surrounded by a basic staining area of outer endocuticle. Fixed in Bouin and stained with iron haematoxylin. Magnification × 825.



M. J. WAY—PLATE I

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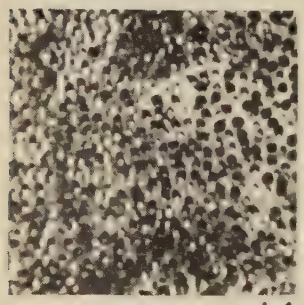

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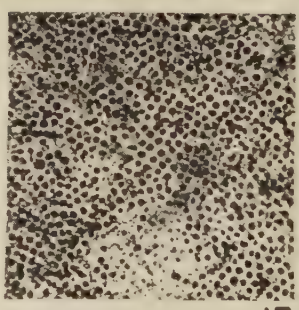

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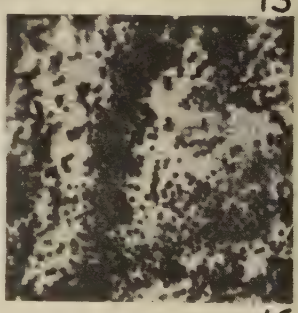

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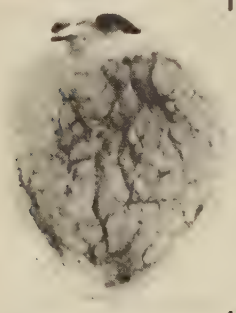

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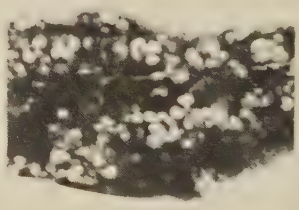

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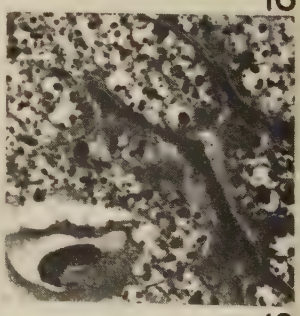

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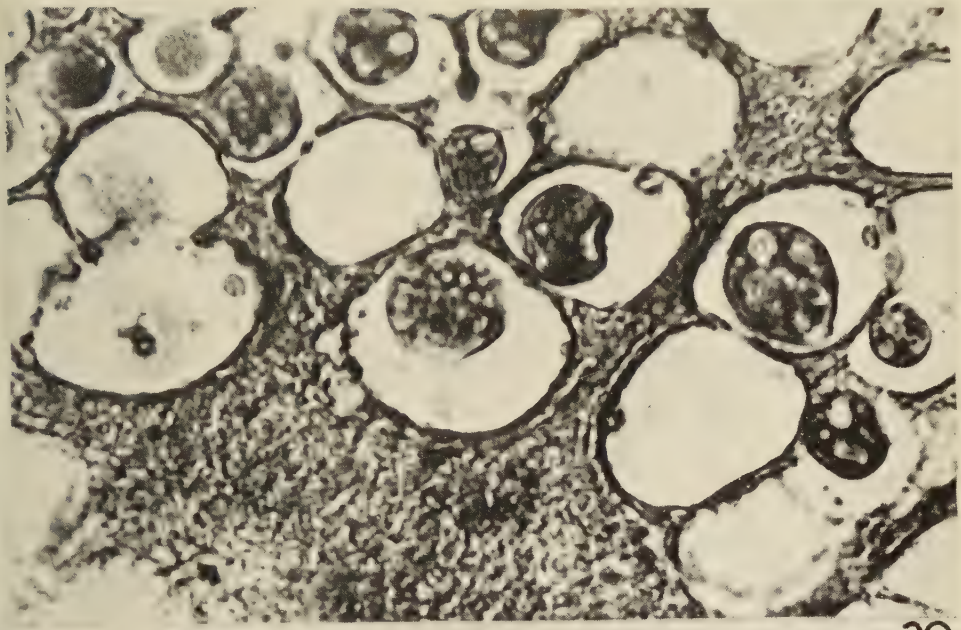
M. J. WAY—PLATE II



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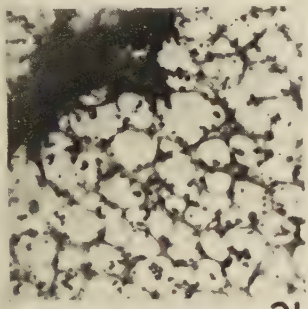

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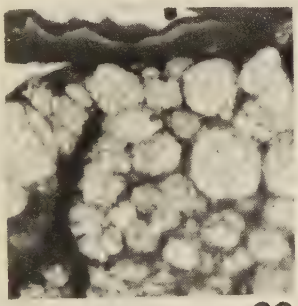

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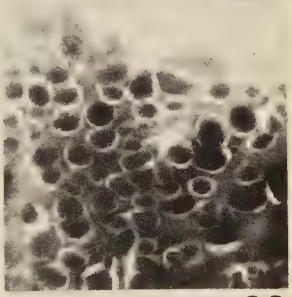

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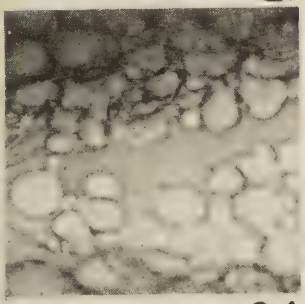

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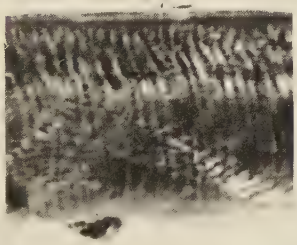

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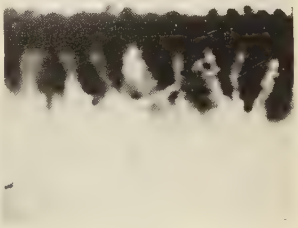

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M. J. WAY—PLATE III

Studies of the Egg of *Bacillus libanicus* (Orthoptera, Phasmidae)

I. The Egg Envelopes

By A. MOSCONA

(*From the Hebrew University, Jerusalem*)

Summary

1. The structure of the eggs of *Bacillus libanicus* (Uvarov) is described and measurements given. Both structure and size were found to vary considerably.

2. The axial orientation of the eggs is not in accord with 'Hallez's law', as the dorsal and ventral sides of the egg and of the early embryo are diametrically opposite to those of the mother.

3. The microscopic structure of the egg envelopes is described. The egg is covered by three main envelopes: a hard, brittle *exochorion* built of seven distinct layers, two of which consist almost solely of calcium salts; a double-layered, membranous *endochorion*; a thin *vitelline membrane*. Layers of the chorion may be summarized as follows:

(i) *exochorion*—tubercular layer, dark layer, lamellar membrane, calcium oxalate layer, intermediate lamella, calcium carbonate layer, shell membrane.

(ii) *endochorion*—outer and inner membrane.

4. In developing eggs the calcium carbonate layer of the exochorion becomes reduced owing to loss of material from its inner surface. Consequently the shell membrane, initially attached to this layer, becomes almost completely detached. The calcium carbonate layer in shells of eggs which do not develop remains unchanged.

Introduction

SINCE the publication of Müller's paper (1825) on the eggs of *Phasma ferula*, there has appeared a considerable number of studies dealing with form and structure in eggs of Phasmidae. Yet, in spite of the large literature, accurate information on the various aspects of this subject is quite scarce and fragmentary.

The first part of the present study deals with the general and microscopic structure of the egg-shell of *Bacillus libanicus* (Uvarov). The species was established in 1924 by Uvarov, who kindly identified specimens from our colony. It is a small phasmid, reproducing bisexually and occurring in certain localities in the Galilean woodlands.

The eggs used in this work were from laboratory-bred specimens. The eggs are laid mainly during the night while the insects feed. They are dropped singly and fall to the ground. A female lays an average of 2·7 eggs in 24 hours, with a total of 300–50 eggs during her lifetime. Embryonic development, including the diapause, lasts for 5–7 months.

Their high mineral content renders the egg-shells hard and brittle, so that

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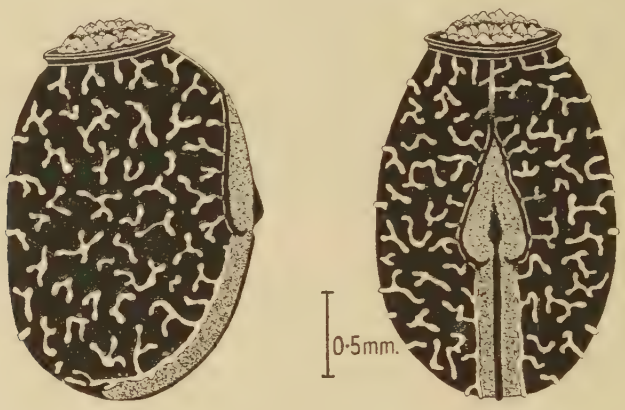
they could be sectioned only after having been treated with nitric acid. The sections were stained either in acid fuchsin and picro-nigrosin or in Mallory's triple stain, which gave good differentiation of the shell layers. Observations on stained material were supplemented by and compared with microscopic examination of untreated shell fragments and of demineralized material mounted in glycerine. This method was also useful in determining the chemical composition of the various shell layers.

General Characteristics of the Egg

Externally the egg is of an opaque black or dark-sepia colour and bears many elongated and branched light- or dark-grey protuberances (Text-fig. 1).



Text-fig. 1. The egg of *B. libanicus* (*a*, lateral surface; *b* convex surface).

When freshly laid, it has a moist and glistening appearance owing to a thin coating of a varnish-like material which dries up in 20–30 minutes, loses much of its sheen, and gradually peels off during subsequent development. This tegument is probably similar in nature to the external coating of the eggs of *Oecanthus* (Ayers, 1884) and *Melanoplus* (Slifer, 1937). Embryonic development induces no externally visible changes in egg colour.

The typical egg has a slightly curved elipsoid form, somewhat compressed laterally. One end is rounded, and the other flattened and covered by a characteristically shaped cap, the *operculum*, bearing a crown-like prominence, the *capitulum*. Near the cap, the egg-shell becomes somewhat constricted into a *neck*, which expands slightly unto a thickened *rim*. On the convex surface of the egg is situated the *micropylar plate* (Text-fig. 1).

The axial orientation of phasmid eggs has never been worked out in detail, which probably accounts for much of the confusion in the terminology used by various authors (Heymons, 1897; Elkind, 1916; Leuzinger, 1925; Cappe de Baillon, 1927). On the other hand, this apparently simple problem actually contains some unexpected difficulties.

Within the ovary the rounded end of the egg is directed posteriorly and the opercular pole anteriorly. The convex surface of the egg is directed towards the dorsal and the flattened surface towards the ventral side of the female.

According to 'Hallez's law' of orientation of insect eggs, the three axes of the egg and of the presumptive embryo are orientated coincidentally with those of the mother. Thus, in our case, the convex surface bearing the micropylar plate would represent the dorsal side of the egg, while the flattened surface would be the ventral one, and, following the usual course of events in insect eggs, should be the site of origin of the embryo (Johannsen and Butt, 1941).

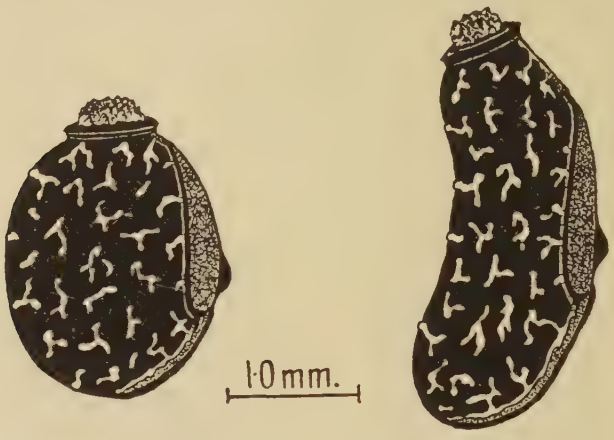
These conditions, which have been found acceptable in most instances, do not fit exactly our case. First, the shape of the dorsal and of the ventral surfaces does not correspond with the condition in other similarly shaped orthopteran eggs, in which the ventral side is convex and the dorsal concave or flattened (Imms, 1942; Johannsen and Butt, 1941). Furthermore, if the convex, micropyle-bearing, side is accepted as dorsal, then the position of the micropyle is unusual as compared with other orthopteran eggs in which, if not polar, it is situated on the ventral and never on the dorsal side of the egg (Roonwall, 1936). Most confusing is the relation between the orientation of the early embryo and that of the egg: the germ disk of *B. libanicus* is formed on the convex surface of the egg, the embryo moving during blastokinesis towards the flattened surface. Upholding Hallez's terminology, this would mean that, in this case, the embryo starts to develop on the dorsal surface of the egg, which would constitute a further exception when compared with the other Orthoptera (Johannsen and Butt, 1941; Imms, 1942), in which group embryonic development always starts on the ventral side of the egg.

Considering, therefore, the shape of the egg, the position of the micropyle, and the situation of the early embryo, it seems evident that the axial orientation of the egg and of the early embryo does not coincide with that of the parent, since there is a 180° difference between their dorso-ventral axis and that of the parent. Exceptions to Hallez's law have been described in the eggs of *Pyrrhocoris* (Seidel, 1924), *Pteronarcys* (Miller, 1939), and of some other insects; whereas in the previously described instances it is the longitudinal axis of the egg or the embryo that does not coincide with that of the parent, in this case the difference involves the dorso-ventral axis.

The most frequent deviations from the typical form of the egg concern the degree of its curvature (Text-fig. 2). In about 1 per cent. of a random sample of eggs the dorsal and ventral sides are equally convex, the eggs having a regular oval outline; these eggs include many viable forms. Some 2 per cent. of the eggs are sharply bent and elongated, with the usually flattened side concave, the shape being that of a slightly curved cylinder. Only rarely do such eggs develop normally.

Size of Eggs

The eggs of *B. libanicus* vary markedly in size, and especially in length. This variability does not affect the characteristic external appearance or structure of the eggs, except in rare, extreme instances. In spite of their being structurally normal, however, many eggs prove wholly or partially incapable of embryonic development. Normal development was found to be restricted to eggs



Text-fig. 2. Abnormal forms of eggs.

between 1·75 and 2·40 mm. in length. Beyond this range, few of the longer and still fewer of the shorter eggs develop to hatching. Functional normality was therefore considered limited to this range, and only eggs within these limits were used in the following measurements.

Table I. *Size of Eggs (in mm.)*

	Min.	*Max.*	$M \pm 3\,m$ *(see below)*	*Standard deviation*	*Coeff. of variation*
Length of egg	1·75	2·40	1·94±0·05	0·167	8·61
Width of egg	1·15	1·50	1·35±0·03	0·089	6·56
Height of egg	1·30	1·70	1·50±0·03	0·098	6·57
Height of operculum and capitulum	0·10	0·30	0·22±0·01	0·037	17·14
Diameter of operculum	0·80	1·05	0·93±0·02	0·062	6·78
Length of micropylar plate, anterior part	0·85	1·55	1·18±0·04	0·144	12·25
Width of micropylar plate, anterior part	0·45	0·75	0·57±0·02	0·068	11·84

Total number of eggs in all measurements—100. Length measured as a straight line between the mid-convex point on the rim of the egg and the posterior pole. Width measured across the widest part of the egg, convex side up. Height measured as a line between the convex and the flattened surfaces of the egg in the plane of the micropylar protuberance.

m is the standard error of the mean (*M*).

Table I summarizes the various egg measurements; the means were calculated as for a normal population distribution. It is apparent from the table that the size of the eggs is very variable; the variation of lengths is most marked, the longest eggs being about 37 per cent. longer than the shortest. The difference between maximal and minimal widths is similar to height variations, the coefficient of variation also being almost equal in both instances. As mentioned above, perfectly shaped although functionally abnormal eggs, of considerably larger or smaller size, sometimes constitute 15 per cent. of the whole batch. The maximal length of such eggs was 3 mm., and the minimal about 0·8 mm. Still smaller aberrant eggs as short as 0·5 mm. also occur, but these consist mostly of misshapen, empty shells.

A considerable variability in size is probably characteristic of phasmid eggs. Heymons (1897) found some normally shaped eggs of *B. rossii* that were only half the average size. Severin (1910) reported that the length of *Diapheromera femorata* eggs varied between 2·0 and 2·9 mm. Contrary to the findings in *B. rossii* the smaller eggs of *B. libanicus* are not necessarily the last ones laid. They may appear at any time during the egg-laying period in spite of uniform feeding conditions. The same has also been observed in *D. femorata*.

The Micropylar Plate and the Operculum

The presence of a micropylar plate is common to all known phasmid eggs. The details of its structure are different and characteristic in each species, so that it has been used as a systematic criterion in this group (Brunner v. Wattenwyl and Redtenbacher, 1906). The micropylar plate of *B. libanicus* is relatively large, elongated, and resembles a spearhead pointing towards the anterior end of the egg (Text-figs. 1, 3).

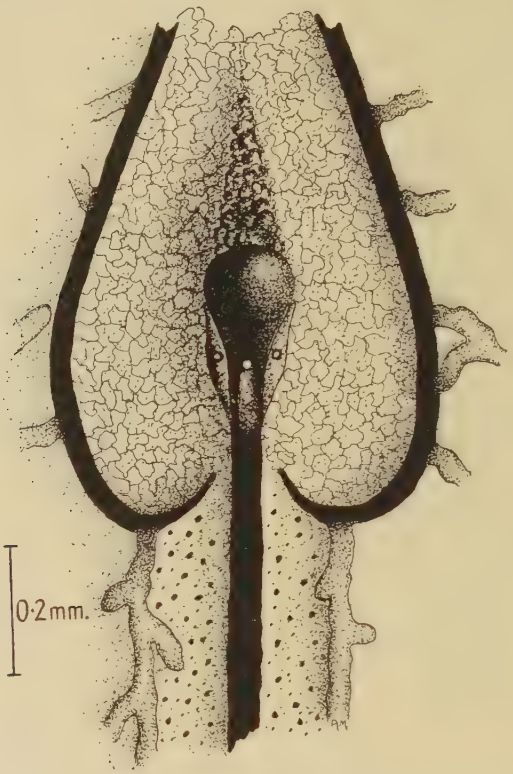
The anterior, broadened part, the plate proper, encloses a dark-coloured prominence at its centre. The posterior elongated part extends towards the posterior pole of the egg. The dark prominence is perforated by three minute apertures of which the central one is the opening of the micropylar canal, while the two lateral ones lead into short blind tunnels which do not perforate the shell. Similar blind tunnels were described in the egg of *Carausius* (Leuzinger, 1925). Their function is unknown.

Table I summarizes a series of measurements of the micropylar plate. The length of the plate varies considerably, and no relation seems to exist between its length and that of the egg. The width of the plate is fairly constant.

The operculum, which according to Sharp (1898) is present in all known phasmid eggs, appears in this case as a flattened lid covering the anterior end of the egg. Most of it consists of a prominent crown-like capitulum with a bowl-shaped concavity in its centre. As in *Carausius* (Leuzinger, 1925) the operculum is fastened to the egg by means of many minute tooth-like ridges fitting into corresponding slits on the inner side of the rim of the egg. It may be easily removed and replaced without injury to the normal functions of the egg. There is no perforation through the operculum at the bottom of the

capitular concavity, such as was assumed by Baehr (1907) to be present in the eggs of *B. rossii.*

Measurements of the diameter and the height of the operculum are summarized in Table I.



TEXT-FIG. 3. Micropylar plate.

THE EGG ENVELOPES

Many of the descriptions dealing with the structure and terminology of the phasmid egg envelopes are mainly of historical interest and need not be mentioned here. More recently, the egg envelopes of *Phyllium crurifolium* have been studied by Henneguy (1890), who distinguished in the hard egg-shell three fused layers, of which the outermost and the median he termed *exochorion* and the innermost *endochorion*. Elkind (1916) in her study of the egg of *C. hilaris*, and Leuzinger (1925) describing the egg of *C. morosus*, termed the whole hard shell *exochorion*. They described an inner, separate, double-layered membrane, which they identified as *endochorion*. They suggested that its inner layer might represent the *vitelline membrane* of other insect eggs. Roth (1916) termed the outer, hard shell of the eggs of *C. morosus*, *capsule*. This term is further applied by Imms (1942) and by Essig (1942) to the external coverings of all phasmid eggs. According to these authorities the capsule of the phasmid

egg is actually an *ootheca* comparable to those of the Blattidae and Mantidae. Thomas (1936) named the external covering of *C. morosus* eggs *chitinous capsule* and the inner, thin membrane (endochorion of Elkind and Leuzinger), *chorion*. He further described the formation of a distinct *vitelline membrane* adhering to the surface of the yolk. In the eggs of *D. femorata*, Severin (1910) was unable to find any structure corresponding to the *vitelline membrane* of Thomas, and applied this term to the thin separate membrane beneath the hard shell.

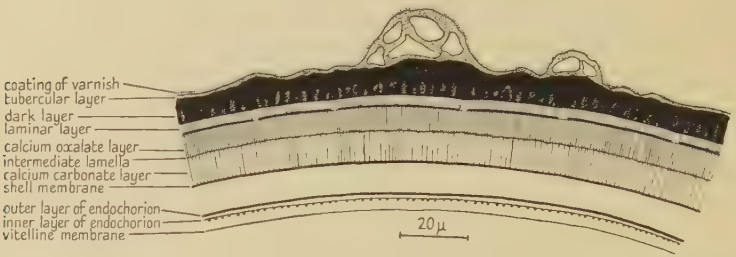
In view of the above confusion of terms it is desirable to define the terms employed in the present description. The term *capsule* refers to the various persisting structures formed outside the egg (or the group of eggs) by the secretions of the accessory or colleterial glands of the oviducts, as, for instance, the oothecae of Blattidae, &c. The term *chorion* represents those of the egg envelopes which are produced by the cells of the follicular epithelium, and does not include any formation added by the accessory glands, the oocyte or the embryo (Slifer, 1937; Beament, 1946). A thickened and hardened chorion is referred to as *shell*. Only the chorionic envelopes are perforated by true micropylar channels. Other envelopes are either unperforated or their perforations are of different origin and function.

The egg of *B. libanicus* is enclosed in three main envelopes: (*a*) an external, hard envelope, 30–50μ thick; (*b*) a median slightly opalescent membrane, elastic and tough, 6–8μ thick, attached to the external envelope only in the micropylar region; (*c*) an inner, delicate membrane about 3μ thick, adhering to the plasmatic layer on the surface of the yolk.

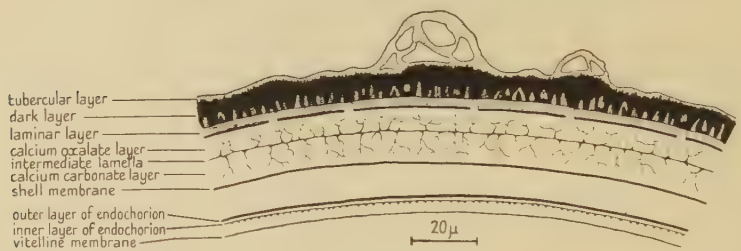
The outer and the median envelopes are perforated by the micropylar canal and their formation is completed inside the ovarian follicle where they are secreted by the follicular cells. We are therefore justified in stating that the eggs are covered from the outside by chorionic envelopes representing the *exochorion* (or *shell*) and the *endochorion* respectively; the terms 'ootheca' or 'capsule' are in this instance unsuitable, the structures referred to being actually the exochorion. The inner envelope is not perforated by the micropyle. It appears when the endochorion is already formed and is secreted by the oocyte. It undoubtedly represents the *vitelline membrane*.

Each of the two chorionic envelopes is composed of distinct layers. The presence of such structures in phasmid egg-shells has been repeatedly recorded, but only Henneguy (1890) and Leuzinger (1925) studied them in detail. Investigating the formation of the egg-shell in Phasmidae, Cappe de Baillon (1933) found that the chorionic layers were formed during successive, periodic phases of secretory activity of the follicular cells, each phase resulting in the completion of new layer, differing structurally from that previously secreted. It was now observed that while the composition and the ultimate structure of the secretion of the *majority* of the follicular cells changes from one secretory phase to the following, other cells may persist in secreting the same substances through two to three phases. This results in the formation of continuous structures extending through two to three layers.

As has already been mentioned, the egg envelopes of *B. libanicus*, like those of many other phasmid eggs, are particularly rich in mineral substances. Practically all of these substances are found in the exochorion (amounting to 38 per cent. of its dry weight), where they are concentrated mainly in two of its layers. The organic components of these layers are restricted to a thin reticular framework, which is revealed after removal of the mineral material by treating the shell with nitric acid. In the other layers the minerals are present in much smaller quantitites, or are totally absent.



TEXT-FIG. 4. Semi-schematic representation of a section through a shell fragment. (Scale is approximate.)



TEXT-FIG. 5. Semi-schematic representation of a section through a shell fragment after treatment with dilute nitric acid. (Scale is approximate.)

Text-fig. 4 represents a section through the envelopes of a freshly laid egg. It should be compared with Text-fig. 5 which represents the appearance of a similar area after treatment with nitric acid; with the mineral material removed, the arrangement of the organic component of each layer is fully revealed.

A qualitative analysis of the shell material demonstrated the presence of calcium, magnesium, aluminium, and potassium in shells. It was further found that the shells contained oxalate, sulphur, and nitrogen, the last two connected with the organic fraction of the shell material. By dissecting small shell fragments under a microscope it was possible to divide the exochorion into two parts approximately on the level of the lamellar membrane (see below), and thus to separate the part containing most of the minerals from the one predominantly organic. With each of these parts various colour reactions were now carried out and localization of the mineral constituents of the shells studied.

The superficial layer of the exochorion forms the greyish tubercles characteristic of the external appearance of the shell; in the intertubercular spaces this tubercular layer is very thin and difficult to distinguish. Inside the tubercles the structure of this layer is sponge-like, enclosing air spaces. The layer consists mainly of a slightly hygroscopic keratin-like substance; it contains small quantities of evenly dispersed inorganic materials which yield a deep-blue colour in reaction with an alkaline solution of quinalizarin, this test being indicative of magnesium (Broda, 1939). The presence of magnesium could be further demonstrated by the red colour obtained with Titian yellow and by the gallic acid test of Crétin (1924) resulting in a yellowish-rose coloration. Tested in the Bunsen flame, fragments of this layer give the colour characteristic of potassium. The presence of this material could be further confirmed by means of the Siena-orange method (Carere-Comes, 1938).

The second layer is much thicker than the previous one and darkly coloured. It consists of an outer compact region sending very short processes into the outer layer, and an inner region with many lightly coloured, irregular spaces rich in inorganic material, which appear more clearly after removal of the minerals. This inorganic material gives the characteristic reactions of magnesium; it also reacts clearly with alizarin red S in ammoniacal solution, giving a red coloration persisting in dilute acetic acid, which indicates the presence of aluminium (Feigl, 1935).

The third layer is in the form of a lamellar membrane consisting of an outer thicker, and an inner thinner portion staining readily with haematoxylin. The membrane is perforated by irregularly spaced minute pores.

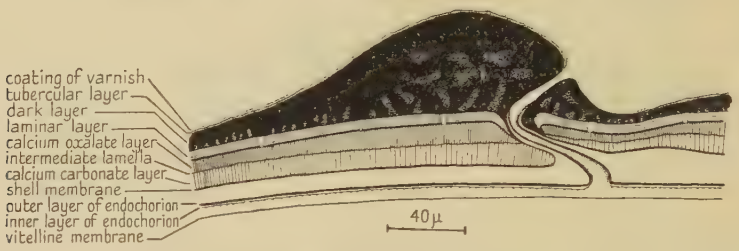
The fourth and sixth layers consist mainly of inorganic materials, supported by an organic reticular framework. The fifth layer has the form of a thin intermediate lamella from which thin ramifications branch out into the two adjoining layers to form their framework. The fourth, fifth, and sixth layers constitute together about half of the total thickness of the exochorion; and it is their high mineral content which is primarily responsible for its rigidity and brittleness. Both layers consist of calcium salts, but, in contrast with the sixth layer which dissolves easily in dilute acetic acid, the fourth layer is insoluble in acetic acid and consists mainly of calcium oxalate. The calcium could be demonstrated by the method of Crétin (1924) (the gallic acid reagent yields in presence of calcium a blue colour). The oxalate could be identified by its ability to decolorize potassium permanganate in solution with dilute sulphuric acid (Feigl, 1935). The sixth layer, somewhat thicker than the fourth, consists chiefly of calcium carbonate; it dissolves readily in weak acids releasing carbon dioxide.

In addition to the inorganic components and the reticular framework, the fourth and the sixth layers contain minute quantities of an unidentified material of waxy consistency. It is evenly distributed throughout these layers and may be extracted with chloroform. It melts at 39° C.

The inner surface of the exochorion is covered by a closely adhering, extremely thin shell membrane already referred to by Leuckart as present in the

eggs of many phasmid species. In freshly laid eggs it can be peeled off only in small fragments but, in the present case, its removal becomes progressively easier with the development of the embryo. It may be easily detached from the shell of the fully developed egg. The shell membrane is attached to the inner surface of the calcium carbonate layer, and the destruction of the latter through immersion in dilute acetic acid results in separation of the membrane.

The thickness of some of the layers of the exochorion varies considerably in the specialized areas of the shell. This is true mainly of the micropylar region, the rim of the shell, and the operculum. Text-fig. 6 represents a section



TEXT-FIG. 6. Semi-schematic representation of a section through a shell fragment in the plane of the micropylar canal. (Scale is approximate.)

through the micropylar protuberance and the micropylar canal, showing relative changes in the various layers in this region. In the rim of the shell and in the rim of the operculum the calcium layers are much thickened, whereas in the rest of the opercular lid they are considerably reduced. The capitular protuberance is formed by proliferations of the tubercular layer.

It has been assumed by several authors (Leuzinger, 1926; Thomas, 1936) that the external covering of phasmid eggs contains chitin, but this assumption was not confirmed in our case as none of the egg envelopes of *B. libanicus* contains any substance giving a positive chitosan reaction after prolonged boiling at 160° C. in a saturated solution of potassium hydroxide (van Wisselingh test).

The *endochorion* consists of two closely adhering membranes; the outer is thicker, tougher, and somewhat opalescent; the inner is thinner, delicate, and bears many small protuberances on its inner surface. In the micropylar region the endochorion forms a short tube penetrating into the micropylar canal of the exochorion (Text-fig. 6).

The exceedingly thin *vitelline membrane* has a homogeneous appearance. It adheres closely to the surface of the yolk from which it may be separated by careful dissection or by a strong jet of water.

Changes in the Exochorion during Development of the Egg

The above description applies to the structure of the envelopes of freshly laid eggs. Since it is generally accepted that insect egg-shells do not change materially during embryonic development, no such changes were anticipated in this case. But, following the observation that the shell membrane becomes

detached from the exochorion in fully developed eggs, the detailed structure of shells of such eggs was investigated with unexpected results. It was found that during development of the embryo a considerable quantity of material is lost from the inner side of the calcium carbonate layer of the exochorion. This loss results in a decrease in thickness of this layer to less than half of its initial size. One effect of this process is the increased transparency of the whole exochorion. Another effect is the loosening of the shell membrane, which, initially attached to the inner surface of the calcium carbonate layer, is released as a result of loss of material from there.

Since no such changes are found in the shells of eggs which do not develop, the possibility of a correlation between these changes and the development of the embryo has to be considered.

ACKNOWLEDGEMENTS

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Studies of the Egg of *Bacillus libanicus* (Orthoptera, Phasmidae)

II. Moisture, Dry Material, and Minerals in the Developing Egg

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(*From the Hebrew University, Jerusalem*)

Summary

1. Weight, moisture content, and mineral content of freshly laid and of fully developed eggs of *Bacillus libanicus* (Uv.) were studied. During development of the embryo the egg-shell loses 19 per cent. of its initial mineral content, while the weight of mineral materials in the embryo increases correspondingly.

2. These changes can be explained only as resulting from transfer of minerals from the shell to the embryo. The mineral materials are derived from the calcium carbonate layer of the shell, which, owing to this loss, becomes thinner during embryonic development.

3. It is suggested that the mechanism of this transfer is based on the production of bicarbonate by the reaction of water and carbon dioxide, given off by the embryo, with the calcium carbonate of the shell.

4. Experimental calcium deficiency in the egg-shells results in a marked lowering of the viability of the embryos; although embryogenesis may sometimes proceed till the hatching stage, the few emerging nymphs survive only for a short time.

5. The possible occurrence of mineral transfer in other phasmid eggs is indicated.

Introduction

IN the preceding paper it was reported that the egg-shell (exochorion) of the stick-insect *Bacillus libanicus* (Uv.) undergoes certain marked structural changes in the course of embryonic development. These changes were shown to have been due to the loss of calcium carbonate from the inner surface of the calcium carbonate layer of the exochorion, with the result that the shell membrane, initially firmly attached to the inner surface of this layer, becomes loosened and easily detachable. As a result of these observations some simple quantitative analyses of the eggs were carried out in order to gain a fuller understanding of the general relations of the main egg constituents. The information thus obtained shed some light on the problems raised by the morphological findings as to the fate of the material lost by the shells and the processes concerned with its removal and transfer.

A series of weight-determinations were carried out with whole eggs, separated egg-shells (i.e. exochorions), and separated egg contents (i.e. yolk-masses or embryos, enclosed in the vitelline membrane and the endochorion), and changes in wet weight, dry weight, and mineral content were studied at different stages of embryonic development.

Weight, Moisture Content, and Ash Content of Eggs

Eggs were cleaned of adhering foreign material and weighed in batches of ten. They were then dried in an oven at 105° C., and their dry weight was

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determined after cooling over anhydrous sulphuric acid. The mineral content was measured by weighing the residual ash after incineration of the eggs.

Table I summarizes the data for wet and dry weights and moisture and ash contents of freshly laid and fully developed eggs. As the total quantity of mineral material remains unchanged during development (Table IV), the data for ash content are included in Table I only. The weight of eggs, like their size, was found to vary considerably in spite of the fact that only normal hatchable eggs were used in these determinations. The extreme values were not included in Table I, but eggs weighing about 3·60 mg. (i.e. about 26 per cent. heavier than the mean weight) are quite frequent; and many abnormally small but structurally complete eggs are also encountered, which weigh about 1·31 mg. (i.e. 45 per cent. of the mean weight). Thus, the heaviest eggs are some 2·7 times heavier than the lightest ones. For comparison it may be mentioned that the extreme weights of eggs of *C. morosus* range between 6 per cent. more and 28 per cent. less than the average value (data from Ling Roth, 1916).

TABLE I. *Mean Weight (in mg.) of a Single Normal Egg*

(a) Freshly laid

	Min.	*Max.*	$M \pm 3m$ *(see below)*	*Standard deviation*	*Coeff. of variation*
Wet weight . .	2·700	3·000	2·845±0·027	0·091	3·19
Dry weight . .	1·403	1·611	1·555±0·027	0·091	5·86
Per cent. of moisture .	43·41	48·41	46·05 ±0·49	1·628	3·53
Weight of ash . .	0·250	0·272	0·258±0·004	0·013	5·04
Per cent. of ash in terms of dry weight . .	15·61	16·89	16·65 ±0·12	0·394	2·37

(b) Fully developed

	Min.	*Max.*	$M \pm 3m$	*Standard deviation*	*Coeff. of variation*
Wet weight . .	2·391	2·521	2·455±0·016	0·053	2·15
Dry weight . .	1·387	1·50	1·455±0·008	0·026	1·78
Per cent. of moisture .	39·21	42·0	40·91 ±0·21	0·70	1·71

m is the standard error of the mean (*M*).

The mean weight of a freshly laid egg is about 2·85 mg., of which 46·1 per cent. is moisture. During development the eggs decrease in weight by 13·7 per cent. This decrease is due mainly to loss of water and partly to the combustion of dry material. Thus the fully developed egg is found to have lost 22·5 per cent. of its original moisture but only 6·4 per cent. of its initial dry weight: 74·4 per cent. of the total loss of weight is therefore due to the evaporation of water and 25·6 per cent. to combustion of dry materials. The comparatively high quantity of residual ash is due to the high mineral content of the egg-shells.

Comparing these data with similar determinations in other insect eggs the following points may be noted. The 13·7 per cent. decrease in wet weight is

greater than in many other instances. Developing eggs of *Bombyx mori* lose 11·6 per cent. of their initial weight (Tichimiroff, 1885); those of *C. morosus* 11·5 per cent. (Leuzinger, 1926); of *Philosamia cynthia* 8·6 per cent. (Bodenheimer, 1931); of *Pachypasa otus* 10·9 per cent. (Moscona, unpublished). On the other hand, the 6·4 per cent. loss of dry weight during development is comparatively low. Eggs of *B. mori* lose 10 per cent. of their weight (Farkas, 1903) and those of *Locusta migratoria*, 20 per cent. (Roonwall, 1936).

Wet and Dry Weights of Separated Egg-shells and Egg Contents

In view of the considerable part contributed by the egg-shell material to the total of the egg components, it seems questionable whether calculations based on weighings of whole eggs actually reveal the fluctuations in weight of the developing eggs in their true proportions. It would be more instructive to determine the weights of the egg-shell and the egg contents separately. This method involves in our case the dissection of large numbers of relatively small eggs, but yields information otherwise unavailable.

It was technically impossible to collect a sufficient quantity of undamaged egg contents without previously drying the eggs. The wet weight of the egg contents could not, therefore, be directly determined, and was calculated from other data. The dry weights of the egg contents are probably somewhat too high owing to the fact that the contents could not always be cleanly separated from the thin membranous endochorion.

Five separate batches of eggs laid by five different females kept under identical breeding conditions were used for these determinations. The shells were removed from freshly laid and fully developed eggs; the shell material was weighed, and its moisture content and dry weight determined. The results are summarized in Table II.

Table II. *Weight (in gm.) of 100 Egg-shells*

(a) Shells of freshly laid eggs

Number of batch	*I*	*II*	*III*	*IV*	*V*	*Average value*
Wet weight . .	0·0667	0·0690	0·0664	0·0693	0·0678	0·0678
Dry weight . .	0·0657	0·0679	0·0652	0·0682	0·0667	0·0667
Per cent. of moisture .	1·62	1·61	1·86	1·67	1·70	1·69

(b) Shells of fully developed eggs

Number of batch	*I*	*II*	*III*	*IV*	*V*	*Average value*
Wet weight . .	0·0565	0·0593	0·0566	0·0600	0·0574	0·0579
Dry weight . .	0·0556	0·0583	0·0555	0·0590	0·0564	0·0569
Per cent. of moisture .	1·59	1·68	1·94	1·72	1·69	1·72

Shells weighed in 5 batches of 100 shells in each; each batch consisted of shells from eggs laid by a different female.

Comparison of Tables I and II reveals the following facts: the shell contributes 23·8 per cent. to the mean wet weight or 42·9 per cent. to the mean dry weight of the freshly laid egg. During embryonic development the shell loses almost 15 per cent. of its initial weight, practically all of this loss being accounted for by a decrease in the quantity of dry material; this indicates a clear quantitative difference between shells of freshly laid and of fully developed eggs.

These results necessitate a revision of several of the conclusions based on weight determinations of whole eggs, as the following analysis of data from Table III shows. The average weight of the shell of a freshly laid egg amounts to 23·8 per cent of the total egg weight. The dry shell material accounts for the remarkably high value of 42·9 per cent. of the dry weight of the whole egg. But in contrast with this considerable share of the shell in the dry weight of

TABLE III. *Weight (in mg.) and Moisture of a Single Egg, the Egg-shell, and the Egg Content (partly calculated)*

	(*a*) *Freshly laid egg*			(*b*) *Fully developed egg*		
	Whole egg	*Egg-shell*	*Egg content*	*Whole egg*	*Egg-shell*	*Egg content*
Wet weight . . .	2·85	0·68	2·17	2·46	0·58	1·88
Dry weight . . .	1·56	0·67	0·89	1·46	0·57	0·89
Per cent. of moisture .	46·1	1·7	59·0	40·9	1·7	52·8
Per cent. of dry material .	53·9	98·3	41·0	59·1	98·3	47·2
Per cent. in terms of wet weight of whole egg .	100	23·8	76·2	100	23·6	76·4
Per cent. in terms of dry weight of whole egg .	100	42·9	57·1	100	39·1	60·9
Per cent. in terms of total moisture in the egg .	100	0·9	99·2	100	1·0	99·0
Per cent. decrease in wet weight during development of embryo . .	..	..	..	13·7	14·7	12·9
Per cent. decrease in dry weight during development of embryo . .	..	..	..	6·4	14·7	0·2
Per cent. decrease in moisture content during development of embryo	..	..	..	22·5	9·1	22·6

the egg, its contribution to the total moisture content, 0·9 per cent., is quite negligible. By subtracting these data from the corresponding weights of whole eggs, the quantities of moisture and of dry material in the dechorionated eggs may be separately estimated. Thus, it is found that the dry material amounts to 41·0 per cent. and the moisture to 59·0 per cent. of the wet weight of the isolated contents of freshly laid eggs. When one compares these results with those based on whole-egg weights (53·9 per cent. dry material, 46·1 per cent. moisture), the differences between them become clear. On the basis of weigh-

ings of whole eggs, one would describe the freshly laid eggs of *B. libanicus* as having a moisture/dry material ratio in favour of the dry materials. This, although correct in general analytical terms, is physiologically misleading, because it does not represent the particular conditions in the egg content, that is to say, in the actual site of embryonic development; there, it is seen, the moisture/dry material ratio is in favour of moisture. The difference, as pointed out, is caused by the methods of measurement and is due to the egg-shell with its low water content and its very high content of metabolically inert material.

A further point revealed by the separate weighings of shells and egg contents concerns the changes in the dry weight of developing eggs. It was found that during embryonic development the egg loses 6·4 per cent. of its initial dry weight. Since this loss results from combustion of storage materials by the embryo, one would expect it to be confined to the egg content and to be fully expressed by a decrease in its dry weight. Yet our weighings yielded quite a different result. During embryonic development the egg content was found to have accounted for only 2 per cent. of the total decrease in the dry weight of the whole egg. Furthermore, the egg-shell alone was found to have lost 14·7 per cent. of its initial dry weight, accounting for about 98 per cent. of the total decrease in the dry weight of the whole egg. This obviously puzzling result would mean that while the egg content, the part directly concerned with embryogenesis, lost only a negligible fraction of dry material during embryonic development, the egg-shell, assumed to be unaffected directly by development, lost a considerable portion of its substance during the same period.

The following determinations of the mineral content of eggs enabled us to clarify this point.

Weight of Minerals in the Egg-shells and Contents

As is evident from Table IV, the total ash content of the eggs remains unchanged during embryonic development, while the ash content of shells decreases from the initial average value of 38·2 per cent. of the dry weight of the shell to 31·8 per cent. Thus, about 29 per cent. of the initial mineral content is lost. This loss accounts for almost all of the decrease in the dry weight of shells of developing eggs; the additional decrease is due to the loss of the outer varnish coating of the shell which peels off gradually. On the other hand, the quantity of ash in the egg content increases during development, reaching a value 12·5 times higher in the fully developed embryo than in the yolk. The only possible interpretation of these weight changes appears to be that the minerals lost from the egg-shell pass into the developing embryo and accumulate there. The increase in minerals in the egg content conceals and compensates in weight for the actual decrease in the dry weight of the developing embryo. The result is the apparently almost unchanging dry weight of the developing egg content in spite of the actual loss of 6·4 per cent. of its dry material.

TABLE IV. *Weight (in gm.) of Ash in Egg-shells and Egg Contents*

(a) Freshly laid eggs

Number of batch	*I*	*II*	*III*	*IV*	*V*	*Average value*
Dry weight of shells	0·0657	0·0679	0·0652	0·0682	0·0667	0·0667
Dry weight of egg contents	0·0886	0·0897	0·0878	0·0896	0·0885	0·0888
Weight of ash in shells	0·0253	0·0255	0·0249	0·0262	0·254	0·0255
Weight of ash in egg contents	0·0006	0·0006	0·0007	0·0007	0·0006	0·0006
Per cent. of ash in shells (dry weight)	38·51	37·55	38·34	38·40	38·17	38·19
Per cent. of ash in egg contents (dry weight)	0·71	0·69	0·82	0·73	0·67	0·72

(b) Fully developed eggs

Number of batch	*I*	*II*	*III*	*IV*	*V*	*Average value*
Dry weight of shells	0·0556	0·0583	0·0555	0·0590	0·0564	0·0569
Dry weight of egg contents	0·0908	0·0895	0·0874	0·0894	0·0885	0·0886
Weight of ash in shells	0·0176	0·0184	0·0180	0·0192	0·0173	0·0181
Weight of ash in egg contents	0·0075	0·0080	0·0073	0·0079	0·0068	0·0075
Per cent. of ash in shells (dry weight)	31·65	31·56	32·43	32·54	30·67	31·81
Per cent. of ash in egg contents (dry weight)	8·26	8·93	8·35	8·61	7·68	8·36

Eggs weighed in 10 batches of 100 eggs in each batch; eggs of both ages were carefully selected to be of closely uniform size.

THE TRANSFER OF MINERALS FROM THE EGG-SHELL INTO THE DEVELOPING EMBRYO

Typical terrestrial eggs, such as those of birds, reptiles, and insects, are fully equipped, at the time of laying, with all the mineral and organic substances needed during the future development of the embryo, and are thereafter closed to the passage of materials other than water and gases (Needham, 1931). It might therefore be supposed that the total amount of minerals required by the embryo is already in the egg content at the time of laying. But it was shown by Tangl and Hammerschlag (1908) that the fully developed avian embryo is considerably richer in calcium salts than the undeveloped avian egg content, and that the additional amount of this mineral in the embryo originates in the egg-shell, whence it is transferred to the egg content. A similar process was also observed in some reptilian eggs by Karashima (1929). Thus, while they are closed to external supply, it is possible, in at least some of these eggs, to increase the initial mineral reserve at the direct disposal of the embryo by drawing on the minerals in the egg-shell.

Reviewing this subject, Needham (1931) raised the question whether there might not occur a similar process in some insect eggs with richly mineralized shells like those of the Phasmidae. This suggestion was based mainly upon the existence of metabolic similarities between insect eggs and other cleidoic eggs. It was further supported by Pantel's observation (1919) that while the Malpighian tubules of the late embryo of the phasmid *Donusa prolixa* contained a considerable quantity of calcium salt crystals, he could detect almost no calcium in the yolk of freshly laid eggs. Pantel interpreted his results by assuming that most of the calcium in the yolk was organically bound and therefore insensitive to his methods of detection. This interpretation was evidently not accepted by Needham, but as no conclusive evidence was then available, the question remained open.

Various observations in the present study furnished the opportunity to reopen this problem and to study the matter in detail. These observations may be summarized as follows. The loss of material from the calcium carbonate layer of the shell of developing eggs, suggested by the resulting structural changes, was quantitatively confirmed by comparing the ash-contents of shells of freshly laid and of fully developed eggs. The mineral content of fully developed embryos was found to be considerably (12·5 times) higher than the initial amount of minerals in the yolk. As any passage of minerals into or out of the eggs can be definitely ruled out in this case, it is concluded that a transfer of calcium carbonate occurs in the eggs of *B. libanicus* from the shell into the developing embryo.

Since the occurrence of this process depends on the development of the embryo, no changes in the distribution of minerals were observed in unfertilized eggs which do not undergo development although they were maintained under incubation conditions identical with those of developing eggs.

As the embryonic development in this species does not proceed at a uniform rate in eggs of equal age, it was necessary during this study to distinguish externally and segregate before dissecting and weighing eggs with fully developed embryos from those retarded in development. Without this distinction, the increase in minerals in the egg contents of 'chronologically ripe' eggs was found markedly lower than in selected fully developed eggs; this was evidently due to the presence in the unselected material of a certain percentage of eggs not fully developed in which the mineral transfer had not yet been completed (Moscona, 1948). Similar is the case with the frequently occurring biennial eggs in which the embryo starts to develop at the beginning of the second year after laying. No changes in the distribution of minerals are observable during the first year, while during the second year these changes are identical with those occurring in other developing eggs.

The question may be raised whether this transfer of minerals is obligatory for the normal development of the embryo. The presence of comparatively large quantities of calcium salts in the form of phosphate (Pantel, 1919), urate, and carbonate in the Malpighian tubules of the late phasmid embryo indicates that the absorbed calcium may be utilized in neutralizing and

eliminating the excess of acids formed in the course of development and thus fills a vital metabolic function. Furthermore, the integument of the freshly hatched nymphs is comparatively richly impregnated with calcium salts.

Some aspects of the functional importance of the process of calcium transfer were studied by observing the effects of experimentally induced calcium deficiency in the egg-shells. Feeding egg-laying females solely on very young leaves of the food plant interferes with the normal mineralization of the egg-shell. The shells of the majority of the resulting eggs are abnormally thin owing to the considerable reduction of the calcium carbonate layer. The ash content in such shells falls to about 10–15 per cent. of the dry weight. The quantity of minerals in the egg content remains unaffected. In about 28 per cent. of such eggs the embryos proceed with development until hatching (as compared with 85 per cent. in normal eggs), but none of the hatched nymphs is viable, most of them emerging with distorted abdomen or legs, unable to move or to feed. The remaining 72 per cent. of eggs contain embryos which have died at various stages of development.

It might be argued that this mortality is due to some nutritional deficiency other than low calcium content in the maternal diet, but it was found that the addition of small quantities of calcium carbonate to the experimental diet led to the deposition of normally developing eggs.

These initial observations therefore suggest that although embryogenesis may proceed to a certain extent in spite of calcium deficiency, the resulting nymphs are unviable.

Another question concerns the method by which the calcium is transferred from the shell into the egg content. Buckner, Martin, and Peter (1925) demonstrated that in the avian egg the carbon dioxide and water, which are given off during incubation, react with the calcium carbonate of the shell producing calcium bicarbonate which diffuses through the membranes into the yolk. The maximal absorption of calcium therefore occurs simultaneously with the period of maximal metabolic activity of the embryo. In eggs of *B. libanicus* the white layer of the shell loses material mainly during the second month of development and during the short post-diapause period, that is, at times when embryonic development progresses most rapidly. It was therefore assumed that a mechanism similar to that of calcium transfer in the avian egg might be operating in this case. Evacuated shells of freshly laid eggs were immersed for 48 hours in twice-distilled water through which carbon dioxide was bubbled. They were then found to have lost material from the calcium layer, acquiring thereby an appearance very similar to that of shells of fully developed eggs. Considered as an accelerated model of some of the conditions acting on the shell during embryonic development, this test supports the suggestion that the mechanism of calcium transfer in *B. libanicus* eggs is initiated by the action of carbon dioxide and water on the calcium carbonate of the shell, as in the avian egg. In the phasmid egg the resulting bicarbonate has then to pass through the shell membrane, the double layered endochorion, the vitelline membrane, and the embryonic envelopes before reaching the embryo.

Nothing definite is known about mineral metabolism of other phasmid eggs, and it is therefore impossible to state whether the process of mineral transfer is of more general occurrence. But various indirect observations, of which Pantel's has already been mentioned, support this view. As early as 1855 Leuckart reported that the shell membrane in the eggs of *Cyphocrania gigas* is more easily detachable from the fully developed than from freshly laid eggs. Similar observations have been made on the eggs of *D. femorata* (Severin, 1910) and of *C. morosus* (Leuzinger, 1926). In *B. libanicus* loosening of the shell membrane indicates removal of adjacent mineral material and its subsequent transfer to the embryo; a similar process may therefore occur also in the other instances in which similar changes in the exochorion have been observed.

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Perinuclear Sudanophil Bodies in Mammalian Epidermis

By WILLIAM MONTAGNA

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With one Plate

Summary

1. In the epidermis of laboratory mammals there are perinuclear lipid spherules which are demonstrated clearly with sudan black. The centre of the larger of these droplets is occupied by a sudanophobe core of varying dimensions.

2. The distribution of the bodies corresponds to the osmiophil elements in comparable tissues, and the structural pattern is similar to that of the Golgi elements in other tissues, as described by Baker and his associates.

3. With the exception of the skin of the monkey, the lipid bodies are negative to the acid haematein test.

Material and Methods

THESE observations were made on the skin and cutaneous appendages in normal mice, rats, hamsters, rabbits, dogs, cats, and monkeys; on the thickened epidermis of biotin-deficient mice, and on that of mice and rabbits which had been anointed with methylcholanthrene. The best results were obtained with tissues fixed in formaldehyde-calcium, postchromed, washed, and imbedded in gelatin, as in Baker's (1946) acid haematein test, and frozen sections were cut at 5μ. Some sections were treated with sudan black, some were stained with acid haematein, and others with nile blue (Cain, 1947*a*). Test preparations treated with sudan IV, oil-blue N, and other oil-soluble dyes give unsatisfactory results for this study. Baker's (1949) improved method for sudan black gave good results.

Unstained frozen sections of similarly fixed tissues were viewed under polarized light, but the lipid bodies were isotropic. Fixed and fresh sections were studied under phase-contrast. Fresh epidermis stained with dilute neutral red in saline was studied under phase-contrast, but the results were uncertain.

Observations

Frozen sections of skin fixed in formaldehyde-calcium, or in formaldehyde-calcium-cadmium, or in other formaldehyde mixtures, show a diffuse sudanophil haze in the epidermis when treated with sudan black. In tissues treated with sudan black after fixation in the acid haematein routine of Baker (1946), or treated with Baker's (1949) improved sudan black method, the epidermal cells contain discrete perinuclear sudanophil granules. These are discernible in all of the apparently healthy epidermal cells (Pl. I, figs. 2–7). In thick epidermis, such as that in the lining of the external auditory meatus, the lipid

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inclusions are distinct in all of the cells below the *stratum granulosum*. In the cells of the *stratum granulosum* they are dust-like and barely distinguishable. Similar sudanophil elements are found also in the cells of cutaneous appendages. They are clear in the cells of the external root-sheath (Pl. I, fig. 6) and in the bulb of growing hairs. In the sebaceous ducts and in the peripheral non-sebaceous cells of sebaceous glands they are well delimited.

In the hypertrophied epidermis of biotin-deficient mice, the perinuclear lipoidal elements are distinct and numerous, as they are also in the cells of

TEXT-FIG. 1. Camera lucida drawing of a group of epidermal cells of the mouse to show the arrangement and structure of the perinuclear sudanophil bodies. Note particularly that the larger vacuolated spherules tend to cluster at one or two poles of the nucleus.

the thickened epidermis of mice and rabbits which have received one application of methylcholanthrene.

The lipoid bodies are mostly in the form of sudanophil spherules which lie close to the nuclear membrane; a few appear as rodlets which resemble the myelin figures of Palade and Claude (1949*a*, *b*). The smaller spherules are simple sudanophil bodies, which are evenly distributed around the nucleus. The larger ones are vacuolated and are usually clustered at one or two poles of the nucleus (Text-fig. 1). Some of the 'vacuoles' are barely visible, but others may be so large that they reduce the lipid part to a delicate peripheral film. Figures which resemble signet rings or demilunes are encountered often.

The other oil-soluble dyes show the perinuclear lipid bodies less clearly than does sudan black. With dilute nile blue (method of Cain, 1947*a*), they appear as vague purplish particles. With acid-haematein, blue-black perinuclear bodies can be seen only in the epidermis of the monkey. In the other animals the test has been consistently negative.

In unstained frozen sections under phase-contrast, refractile perinuclear bodies can be seen, but not without some difficulty. Since structural details

cannot be seen, it is not certain that these correspond to the lipid bodies described above.

After treatment with sudan black, the lipid bodies are decolorized in sections which are rinsed too long in 50 per cent. alcohol. Rinsing in 70 per cent. alcohol for more than a few seconds completely removes the sudan. However, after such treatment they can be coloured again with sudan.

Discussion

In recent years Hirsch (1939), Baker (1944, 1949), Cain (1947, 1949), Thomas (1948), and Palade and Claude (1949*a* and *b*) have presented evidence that the classical Golgi net is an artifact which develops from a complex of lipoidal spherules. Some of these spherules contain sudanophobe (or osmiophobe, or chromophobe) kernels, and there is a suggestion that these vesicles mark in some way the activity of the Golgi bodies. (Gatenby and Moussa, 1949*a*, *b*, and Gatenby *et al.*, 1949, are opposed to these views, and reinterpret the Golgi complex as a canalicular system with which the lipid bodies are associated.) The present observations on the sudanophil bodies in mammalian epidermis seem to agree with the findings of Hirsch and the other authors named with him. The lipid elements described here correspond to the osmiophil or argyrophil bodies in the epidermis, and they are similar to the spheroidal system described by the above-mentioned authors (Pl. I, figs. 2–7). I am not prepared to say whether they represent the entire Golgi element or only a part of it.

There is no specific information concerning the chemical composition of these elements except that they are lipoidal. Since only the epidermis of the monkey gave a positive acid-haematein test (Cain, 1949, mentions that he was able to show the Golgi elements with the acid-haematein test in the Malpighian layer of the guinea-pig skin), it is possible that if the lipid bodies in the other animals studied contain phospholipids, they are either present in small amounts, or they are in combination with other substances which inhibit the acid haematein test. These discrepancies could be explained by Worley's (1946, p. 36) statement that the 'Golgi bodies of no two kinds of cells can be expected to possess exactly the same total chemical composition'.

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EXPLANATION OF PLATE I

All sections are from tissues fixed in Baker's acid-hematein routine, cut at 5 μ and treated with sudan black. Magnification approximately $\times$ 1,800.

FIG. 2. Epidermis of mouse. The heavy sudanophil tab at the proximal pole of one of the nuclei is actually composed of a series of vacuolated spherules (cf. Text-fig. 1).

FIG. 3. Ditto. One of the larger spherules is guttate. Actually this surrounds a vacuole not visible in the photograph.

FIG. 4. Epidermis of rabbit. Note the grouping of larger spherules.

FIG. 5. Ditto. Note vacuolated spherules basal to the nucleus.

FIG. 6. External root-sheath of rabbit. Lipid bodies indistinguishable from those of other epidermal cells.

FIG. 7. Epidermis of monkey. Heavy accumulation of lipid bodies in the basal cells.

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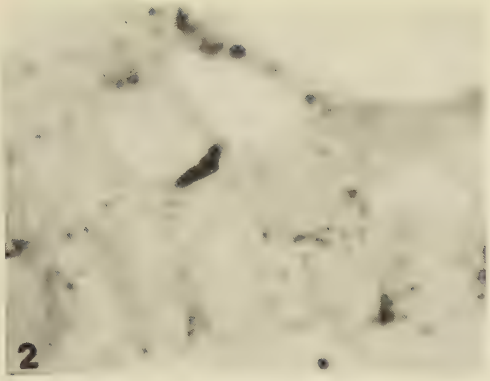

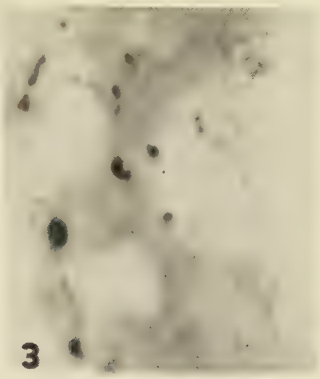

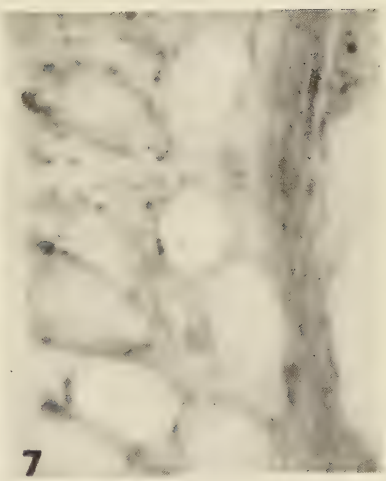

W. MONTAGNA—PLATE I

Alkaline Phosphatase in Protonephridia of Terrestrial Nemertines and Planarians

By J. F. DANIELLI AND C. F. A. PANTIN

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With one Plate

Summary

1. Application of the Gomori method to sections of the terrestrial nemertine *Geonemertes dendyi* shows that there is no alkaline phosphatase in the flame cells or in the distal glandular canal of the protonephridia. But the proximal ciliated convoluted canal and the branching ciliated terminal ducts are rich in this enzyme.

2. Sections of the terrestrial triclad *Rhynchodemus terrestris* show the presence in the parenchyma of numerous ciliated convoluted canals resembling those of the nemertine.

3. A study of the protonephridial system of *R. terrestris* shows that this consists of numerous flame cells connected by branching unciliated ducts with the phosphatase-rich ciliated convoluted canals. The latter connect with unciliated distal canals which probably connect with the numerous exit ducts. These exit ducts are apparently confined to the ventral surface, particularly along the ciliated creeping sole.

4. The convergence between these nemertine and turbellarian protonephridia, and their analogy with the differentiated tubule system of the vertebrate kidney, are noted.

IN an earlier paper (Pantin, 1947) it was shown by one of us that the excretory system of the terrestrial nemertine *Geonemertes dendyi* Dakin consisted of many thousands of protonephridia in a layer immediately beneath the muscular body-wall. The whole surface is covered by numerous minute nephridial openings. Each opening communicates by an efferent duct with a convoluted glandular canal with thick walls of radially striated protoplasm. Passing back, this glandular part suddenly opens into a highly convoluted end-canal from whence terminal branches serve the numerous flame cells. The end-canal and its branches have relatively thin walls and are ciliated, which the glandular canal is not. The differentiation of the system is seen in Pl. 1, fig. *a*.

There is evidence that the protonephridial system is concerned with water regulation, and the histological differentiation of sections of the canal system call to mind the differentiation of the kidney tubule in Vertebrates. In these a striking feature is the rich supply of alkaline phosphatase localized in the proximal tubule leading from the glomerulus and its absence in the distal tubule. Accordingly it seemed of interest to investigate the distribution of this enzyme in protonephridia.

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Methods

For ordinary micro-anatomical studies, paraffin sections were stained with Mallory, and with Masson's haematoxylin–ponceau–light-green method.

For the detection of alkaline phosphatase Gomori's (1939) method was used. The worms were fixed for 2 hours in ice-cold 80 per cent. ethyl alcohol. They were then passed through three 20-minute changes of absolute alcohol and two of cedar oil, followed by two changes of 52° paraffin wax of 10 minutes each. The subsequent treatment followed the plan given by Danielli (1946). The sections were incubated for 2 hours in the presence of the glycerophosphate substrate at 27° or 37° C. The presence of the enzyme was rendered evident in the usual way by the ultimate deposition of black cobalt sulphide. The sections were counter-stained with light green.

Controls, in which the glycerophosphate substrate was omitted, showed no blackening except where the calcareous bodies in the ectoderm reacted directly with the cobalt sulphide.

Results with *Geonemertes dendyi*

Pl. I, fig. *c*, shows a tangential section through the nephridial layer just below the dermal musculature. The presence of abundant alkaline phosphatase in the whole end-canal system, in its convolutions and in the branched end ducts, is very evident. Controls (Pl. I, fig. *d*) show no deposition of the sulphide.

A careful study of the distribution of the enzyme shows that it is confined to the end-canal system and is quite absent both in the flame cells and in the glandular canal. This is seen in Pl. I, in which fig. *b* shows the distribution of the enzyme in the same nephridium as that figured in its entirety in fig. *a*.

Results on *Rhynchodemus terrestris* (O. F. Müller)

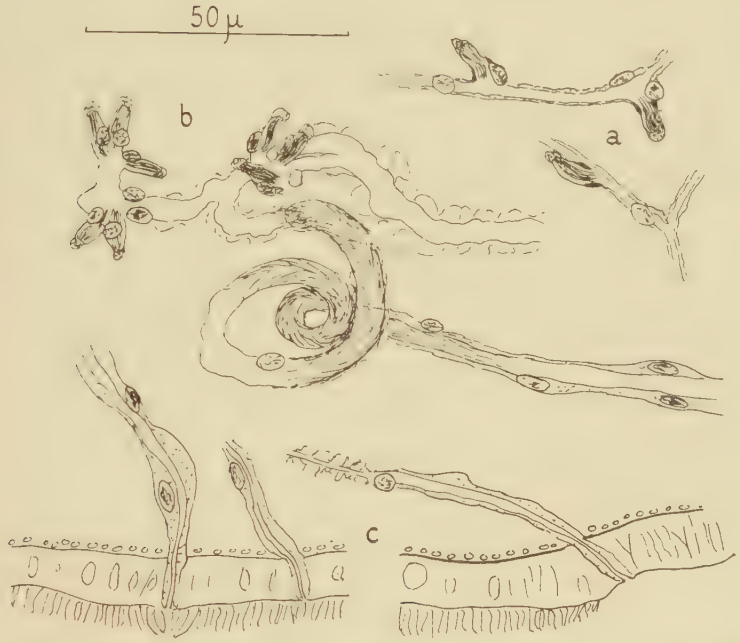
This worm lives in the same humid environment as *G. dendyi*. Despite the phyletic distinction between nemertines and Turbellaria there are certain functional parallels in their grade of organization. Both possess a protonephridial system, though little is known of the organization of this in terrestrial triclads.

Sections of *R. terrestris* treated by the Gomori method at once brought to light the existence of localized convoluted ciliated tubules heavily stained with cobalt sulphide owing to the presence of large amounts of the enzyme. Pl. I, fig. *e*, shows such a convoluted canal. It is noteworthy that the black sulphide is deposited not only in the wall of the canal, but also strongly upon the cilia. A similar deposition upon the cilia is to be seen in the convoluted canals of *G. dendyi*, though it is less marked. Pl. I, fig. *f*, shows a similar convoluted tubule in the control.

Similar convoluted canals can be seen with active ciliary movement in the

tissues of *R. terrestris* during life. They, and the flame cells, are the only parts of the nephridial system visible in living tissues.

These convoluted ciliated ducts, rich in the enzyme, strongly resemble those of *G. dendyi*. They are, however, scattered all through the parenchyma and are not confined to a sub-dermal layer, as is the case in the latter. No other



Text-fig. 1. Protonephridia of *Rhynchodemus terrestris*. (*a*) Isolated flame cells and end-canals. (*b*) Groups of flame cells opening into end-canals. End-canals opening into convoluted ciliated canal. Ciliated canal opening into final ducts. (*c*) Final ducts opening into exit canals through ventral surface.

parts of the excretory system are rich in the enzyme, not even the terminal branches leading to the flame cells. Because they are isolated, connexion of these enzyme-rich coiled ducts with the excretory system must be established by other methods.

The Protonephridia of *R. terrestris*

The excretory system of these terrestrial triclads is far harder to determine than that of *G. dendyi*. Unlike the latter, the system is not confined to a definite layer, but is diffused throughout the parenchyma. Further, although the system is extensive, the flame cells are small and the ducts narrow; and the ill-defined structure and thinness of the walls of the ducts make them hard to distinguish in the complex pattern of cell types and other structures in the parenchyma. Once observed, however, the different elements of this

system can be detected and are seen to be abundant at all points. But the connexion between the elements is difficult to determine except in an occasional lucky section.

The flame cells are about 10μ long and 4μ broad (Text-fig. 1*a*). They possess a single lateral nucleus, and the ciliary flame arises from a simple cap of protoplasm. The lower part of the flame cell is apparently supported by some strengthening material which stains with iron haematoxylin. This recalls the supporting structures which Schröder (1918) found in the flame cells of *G. palaensis*, though the elaborate ribs of the latter have no counterpart in these triclad flame cells.

The flame cells open singly and in groups into branched terminal ducts the walls of which are often almost impossible to distinguish. In favourable sections, particularly when using Masson's iron haematoxylin–ponceau–light-green method, the sudden passage of these ducts into ciliated convoluted canals, identical in structure with those seen with the Gomori method, is seen (Text-fig. 1*b*). From these ciliated convoluted canals a duct with thicker glandular walls descends through the parenchyma towards the ventral surface (Text-fig. 1, *b* and *c*). The ducts are numerous and they run a long way. Like the end-canals they are very difficult to trace through the varied structures of the parenchyma. The path of any single duct has not yet been followed by us continuously to the exterior, but ducts of similar structure and running in the same direction are found connected to exit canals. These exit canals are very numerous, opening on to the ventral surface, particularly on the curious ventral ciliated sole along which the animal glides (Text-fig. 1*c*).

Conclusion

These results show a striking convergence between the protonephridia of a species of terrestrial nemertine and a terrestrial triclad. In both there are numerous flame cells, though in the triclad these are not confined to the sub-dermal layer. In both, the end-canals from these lead to a convoluted ciliated canal rich in alkaline phosphatase, which is absent in the rest of the system except that both ciliation and the phosphatase extend back into the terminal ducts in *G. dendyi*. Distally, beyond the convoluted canal the histology changes. In *R. terrestris* there is a thicker walled duct. In *G. dendyi* there is the curious glandular canal. Finally, in both, the excretory canals open to the exterior by very numerous minute efferent ducts. These are distributed over the whole ciliated surface in *G. dendyi* whilst in *R. terrestris* they are found only in the neighbourhood of the ventral strip to which the ciliation of these animals is confined.

We have made no studies of the extent to which our studies of phosphatase are complicated by diffusion phenomena. Consequently we cannot claim that the detail of the distribution of cobalt sulphide observed corresponds to the detail of phosphatase distribution. All that is claimed is that there is a high concentration of alkaline phosphatase in the end-canal system of the protonephridia of *G. dendyi*, and in the convoluted tubues of *R. terrestris*. We can-

not, for example, be certain that the blackening of the tubule cilia indicates that the cilia in this region are rich in phosphatase.

With the above reservation there is an interesting *functional* resemblance between the nephridia studied here and the nephrons of fish, amphibia, and mammals. In the fish having glomerular kidneys, in amphibia, and in mammals one unit, the glomerulus, produces a passage of fluid into a tubule system (see review, Smith, 1937). This fluid passes down the proximal and distal tubules, and its composition is modified during its passage by secretory activity of the tubule cells. The tubules proximal to the glomerulus have brush borders which are rich in alkaline phosphatase (Gomori, 1939; Lorch and Danielli, 1951), and even in the fish having aglomerular kidneys the borders of the cells of the tubules corresponding to proximal tubules are rich in phosphatase. In the same manner, the protonephridia are organized so that one type of unit, the flame cell, produces a passage of fluid into a tubule system: the cells of the part of the tubule system more or less proximal to the flame-cell system are distinguished from other parts of the nephridia by being rich in alkaline phosphatase. It seems very probable that in both nephrons and protonephridia the phosphatase is connected in some way with the process of modifying the composition of the fluid passing down the tubules.

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EXPLANATION OF PLATE I

Fig. *a*. *Geonemertes dendyi*. Camera lucida drawing of an entire protonephridium.

Fig. *b*. *Geonemertes dendyi*. Alkaline phosphatase distribution in end-canal system, and its convolutions, from the same specimen. Note absence of enzyme in flame cells and in final glandular canal. (Incubation 2 hours at 37° C.)

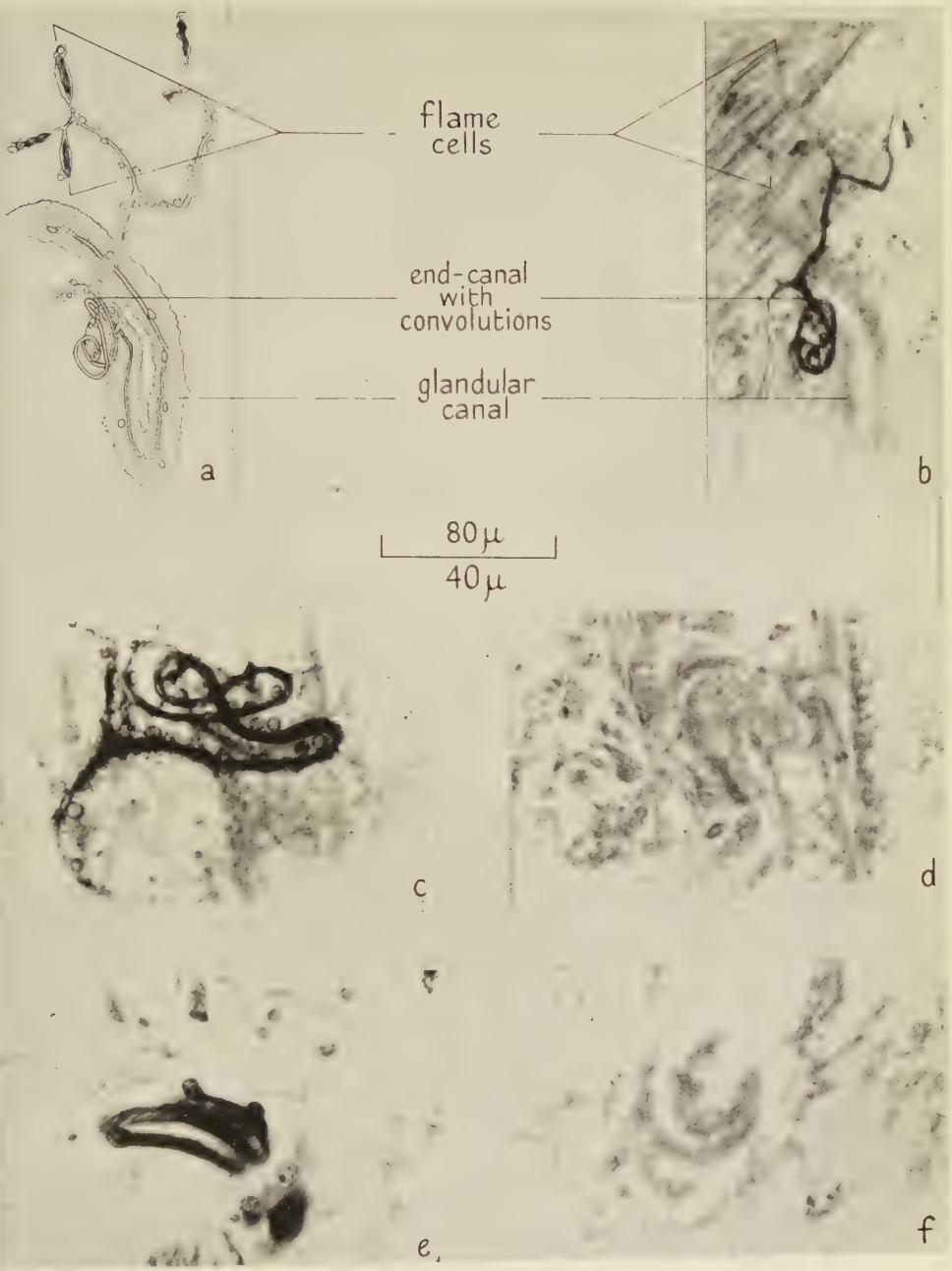
Fig. *c*. *Geonemertes dendyi*. Alkaline phosphatase in end-canal system. (Incubation 2 hours at 27° C.)

Fig. *d*. *Geonemertes dendyi*. Control, showing absence of blackening in convoluted end-canal.

Fig. *e*. *Rhynchodemus terrestris*. Alkaline phosphatase in ciliated convoluted tubule. (Incubation 2 hours at 27° C.)

Fig. *f*. *Rhynchodemus terrestris*. Control showing absence of blackening in ciliated convoluted tubule.

For Figs. *a* and *b*, bar is 80 μ. For Fig. *c–f*, bar is 40 μ.



J. F. DANIELLI and C. F. A. PANTIN—PLATE I

On the Cytochemical Demonstration of Aldehydes

By J. F. DANIELLI

(*From the Department of Zoology, King's College, Strand, W.C. 2*)

SUMMARY

Techniques are discussed for the cytochemical demonstration of (*a*) free aldehydes, (*b*) aldehyde acetals, and (*c*) aldehydes formed by oxidation.

IN studying the distribution in tissues of substances which react with reduced fuchsin, it is frequently necessary to distinguish between (*a*) substances which react as though they were free aldehydes; (*b*) substances which react as though they were aldehyde acetals; and (*c*) substances which react as aldehydes as a result of oxidation of precursors in the tissue.

In a recent paper on the plasmal reaction, A. J. Cain (1949) remarks on one of my papers on liver aldehydes (Danielli, 1949): 'The technique proposed (p. 68) is open to serious objection in that the fixative used (and recommended, p. 70) contained acetic acid, and that the time of fixation is given as not less than two hours and not more than five days. . . . This suggests that it was the pseudoplasmal reaction that was studied, not the true plasmal reaction.' Thus Dr. Cain supposes (A) that by using an acid fixative I failed to distinguish between free and acetal aldehyde, and (B) that the duration of fixation introduced artefacts due to oxidation. Neither supposition is correct.

With reference to (A): Dr. Cain is referring to a part of my paper in which I considered only *free* aldehyde, but on p. 68 I stated: 'In section 7 of this paper a procedure is given which distinguishes between (*a*) free aldehyde groups, (*b*) aldehyde groups bound as acetals, and (*c*) aldehyde groups liberated by oxidation', and on p. 73 I gave a detailed procedure: 'It may be necessary at times to discriminate between (*a*) free aldehydes, (*b*) aldehydes present as acetals, and (*c*) aldehydes liberated by oxidation. This can be done as follows, *after fixation with neutral formaldehyde solution*:[1]

(1) Expose to reduced fuchsin, omitting treatment with mercuric chloride or hydrochloric acid. This demonstrates free aldehyde only. Care must be taken to avoid using an acidic fuchsin solution.

(2) Use of cold N/10 HCl (prior to reduced fuchsin) will show both free and acetal aldehyde, but if the free aldehyde is converted to oxime (before the exposure to acid) only acetal aldehyde will be demonstrated.

(3) Treatment with cold N/10 HCl, followed by oxime formation, then by (oxidizing agents such as) mercuric chloride, shows only aldehyde formed by oxidation.'

[1] Italics not in original.

[Quarterly Journal of Microscopical Science, Vol. 91, part 2, June 1950.]

It is clear that I recommend a neutral fixative where aldehydes of types (*a*), (*b*), and (*c*) are to be distinguished.

With regard to (B): two steps in my procedures exclude the possibility that I was confusing an aldehyde reaction due to oxidation (the pseudoplasmal reaction) with aldehyde present as free aldehyde or acetal (the plasmal reaction). These steps were:

1. The variation of time of exposure to fixative. The pseudoplasmal reaction increases in extent with increased duration of fixation. Under my conditions there was no such change in the intensity of reaction of liver aldehydes over several days. Maximum intensity of reaction is given after a brief exposure to formaldehyde.
2. The simplest method of avoiding a pseudoplasmal reaction is to fix *in vacuo* when no oxygen is available for oxidation. This procedure was always used as a check method.

Thus it is clear that my results are not invalidated by artifacts due to oxidation.

Dr. Cain has suggested, to minimize oxidation, 'the use of very small pieces of tissue which are exposed while fresh to the action of Schiff's reagent (reduced fuchsin) both with and without mercuric chloride, and then plunged into formaldehyde'. This technique I tried two years ago but abandoned for two reasons: in the first place fixation of the tissues under investigation was very poor by this method; and, secondly, no way was found by which the magnitude of diffusion artifacts could be checked if the tissue is exposed directly to reduced fuchsin. A cytochemical method which does not permit estimation of the magnitude of diffusion artifacts is useless.

With reference to the pseudoplasmal reaction: in many tissues, as various workers have found, there is a marked increase in apparent free aldehyde with the passage of time after death. This may be very marked with, for example, Walker sarcoma tissue. It may well be that this reaction, due to oxidation, may be produced by ethylene oxides or other compounds of lipoids and oxygen. But there is no evidence so far available that substances such as ethylene oxides or hydroperoxides of fats normally occur in significant amounts in living tissue. In this respect the pseudoplasmal reaction differs very markedly from the reactions for free aldehyde and acetal aldehyde: the later compounds appear often to be major components of the lipoids of living tissues.

Finally, Dr. Cain remarks, 'it does not appear that control sections were used'. I can assure him that control sections were in fact used in all experiments as a matter of routine.

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A New Method for injecting the Tracheae and Tracheoles of Insects

By V. B. WIGGLESWORTH

(*From the Agricultural Research Council Unit of Insect Physiology, Department of Zoology, Cambridge*)

With two Plates

SUMMARY

A simple method of evacuating and injecting the tracheal system is described.

The injection fluid consists of copper, cobalt or lead naphthenate in light petroleum ('white spirit'). This is converted to the black metallic sulphide by exposing the insect to sulphuretted hydrogen after injection.

Tracheae and tracheoles are revealed. After fixation the tissues may be cleared and mounted in Canada balsam or sectioned and stained.

Methods of bleaching dark coloured insects after injection are described.

FROM time to time during the past twenty years I have sought to devise a method of obtaining permanent injections of the tracheal system of insects. This work has now reached a stage at which it may usefully be published.

The customary method employed for studying the anatomy of the tracheal system is to clear the insect in glycerol or glycerol jelly (Kielich, 1918; Landa, 1948), or to inject with Sudan III in oil and mount in glycerol (Gäbler, 1933). Lehmann (1924) has described a method of filling the larger tracheae with 'paraffin asphalt', the soft tissues being afterwards dissolved with potash, and Krogh (1917) used a similar method employing a turpentine extract of alkanna root mixed with paraffin, beeswax, and colophony to melt at 40° C. Tichomirov (1924) succeeded in injecting the main tracheae of large caterpillars with Prussian blue which was subsequently precipitated with alcohol; and Kirk (1924) obtained results with carmine in metagelatin. Hagmann (1940) injected the tracheae and many of the tracheoles of the cockroach with trypan blue in a wetting agent and was able to prepare sections of the material.

With the possible exception of that of Hagmann, none of these methods reveals the tracheoles satisfactorily or permanently. By exposing the insect to the vapour of osmium tetroxide before clearing in glycerol the entry of fluid into the tracheal endings can be delayed to some extent, and this facilitates the observation of the air-containing capillaries (Koeppen, 1921). The finest branches will sometimes stain with the silver impregnation methods of Golgi and Cajal (Cajal, 1890; Holmgren, 1907; Prenant, 1911; Morison, 1927); but these procedures give very uncertain results, sometimes revealing intra-cellular networks which are only doubtfully connected with the tracheal system (Athanasiu and Dragoiu, 1914; Montalenti, 1926).

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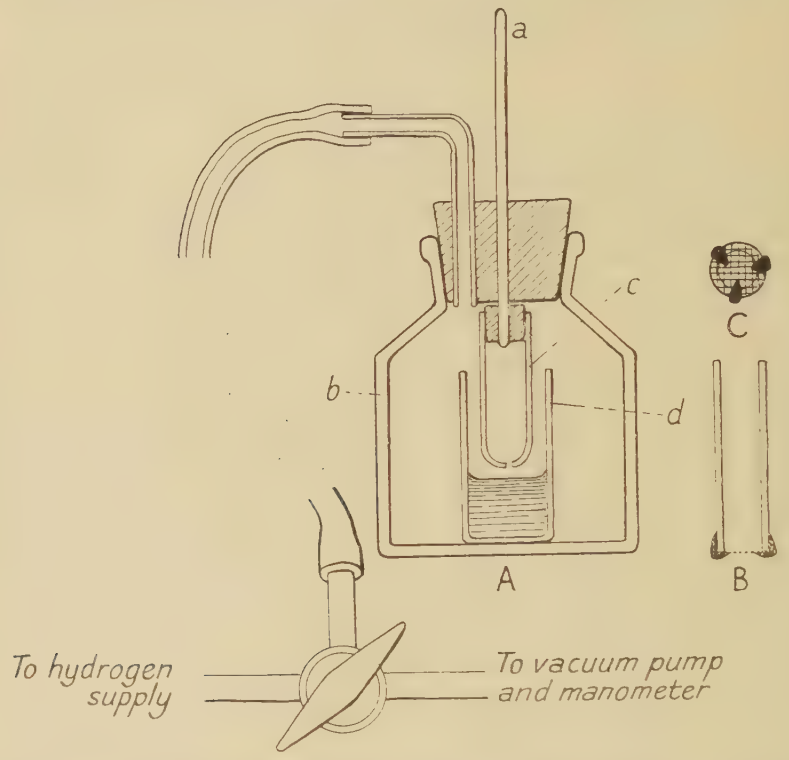
Method of Filling the Tracheal System

In order to fill the tracheae rapidly and completely it is desirable to evacuate all the air from the system before immersion in the injection fluid. A simple method of doing this is shown in Text-fig. 1*a*. A glass rod *a*, lubricated with glycerol, passes through the rubber bung of a small wide-mouthed bottle *b*. The insects to be injected are placed alive in the container *c*, which is a round-ended glass tube with a small hole drilled in the bottom or, alternatively,



Text-fig. 1. A, apparatus for evacuation and injection of tracheal system; explanation in text. B, container for insects consisting of glass tubing with disk of fine metal gauze secured to lower end by adhesive wax. C, the same seen from below.

a piece of glass tubing closed below by a disk of fine metal gauze secured to the end by three small lugs of adhesive wax (B.D.H. Sira wax) (Text-fig. 1, *b* and *c*). The tube *c* is closed by a bung into which the rod *a* is inserted and which has a slit at the margin to allow the passage of air. The tube *c* is thus suspended above the vial *d* containing the injection fluid. The bottle *b* is connected to a three-way tap, one arm of which leads to a mercury manometer, trap, and filter pump, the other to a bladder of hydrogen.

The bottle *b* is evacuated to a pressure of 10–15 mm. of mercury. (If it is reduced below this by the use of a high vacuum pump the injection fluid is apt

to boil excessively.) The pressure is then restored to that of the atmosphere by letting in hydrogen. Evacuation is repeated and hydrogen once more introduced. Evacuation to 10 mm. Hg is carried out again, and the insects are immersed in the fluid by pushing the rod *a* through the bung so that the container *c* fills from below. The pressure is then raised gently but rapidly by the removal of the hydrogen bladder and the admission of air.

Injection of Oil-soluble Dyes

It was thought that by the use of the intensely coloured dye sudan black B or of oil deeply blackened with osmium tetroxide it might be possible to make the tracheoles visible. Mobile injection fluids were made by saturating with sudan B a mixture of equal parts of olive oil and 'white spirit' (petroleum in the boiling range 150–90° C.) and by adding 0·2 gm. of osmium tetroxide to 5 c.c. of the same oil mixture.

Very dark coloration of the main tracheal branches in *Drosophila* was secured, but the tracheoles were so pale as to be scarcely visible. Satisfactory fixation without precipitation of sudan B was not obtained; and, in any case, the slightest injury to the tubes led to the escape of the contents into the tissues. This line of approach was therefore abandoned.

Injection with Metallic Sulphides

The ultimate objective was to obtain an injection after which the insect could be fixed, cleared in xylene, and mounted in Canada balsam or studied in sections. The idea was conceived of injecting in solution a substance which would then react with some chemical (preferably a gas), subsequently applied, to form an opaque precipitate. After many trials it appeared that the most hopeful procedure would be to use the salts of some metal which forms a black sulphide on exposure to sulphuretted hydrogen.

This would present no difficulty if aqueous solutions of such salts could be made to enter the tracheal system. Mixtures of lead acetate, cobalt nitrate, &c., were tried, not only in such familiar wetting agents as bile salts, saponin, Turkey red oil, &c., but in a very wide range of proprietary detergents. In no case would the solution enter the tracheal system of the small insects used for trial (*Drosophila*, *Xenopsylla*, small nymphs of *Cimex*). These mixtures were exceedingly active in wetting the surface of the cuticle, but the tracheae behave as though they are lined with free wax.

Solutions of cobalt nitrate in moderately long-chain alcohols or fatty acids (amyl or butyl alcohol, butyric acid, &c.) will readily fill the tracheal system, but they diffuse at once into the tissues, so that the entire insect is blackened by the sulphuretted hydrogen.

It is clear that an oily solution is necessary. By the use of intermediate solvents it is not difficult to obtain a high concentration of cobalt nitrate in white spirit; for example by the use of Lubrol W, Lubrol MO, and Dispersol A (Imperial Chemical Industries Ltd.). But these mixtures require the addition

of a small amount of amyl alcohol to render them entirely clear, and this leads to rapid entry into the tissues. A number of fairly satisfactory mixtures were obtained with the aid of the series of cetyl ethers of polyethylene glycol with graded properties, described in an earlier paper (Wigglesworth, 1945), and some excellent injections of the tracheoles in the flea and in *Drosophila* were secured. But these materials are not available commercially, and in any case they give uncertain results owing to the more or less rapid escape of the salt into the tissues.

Search was therefore made for oil-soluble salts with suitable properties. Copper oleate proved fairly successful. This will not dissolve directly in white spirit, but 1 part by volume of copper oleate dissolved by warming in 0·5 parts of ethylene glycol mono-oleate (Glyco Products Co. Inc.) mixed with 1·5 parts of white spirit gave a mobile solution which yielded some excellent preparations of the larger tracheal branches. Pl. I, fig. 1, shows the tracheae and air-sacs in the leg of *Blattella*. Apparently the wetting agent spread ahead of the copper salt in the finest tracheal capillaries, and these were poorly revealed. Much better results were secured with a preparation of copper naphthenate containing 10 per cent. of metal, which will dissolve directly in white spirit and gives a sufficiently mobile solution when mixed with twice its volume. But in the tracheoles the copper sulphide appears pale brown and does not show up very well.

The best results have been got with cobalt naphthenate containing 8 per cent. of metal. This is a deep purple semi-solid tar which gives a highly mobile solution when mixed with an equal volume or with two volumes of white spirit. On treatment with hydrogen sulphide it gives an intensely black even precipitate which can be followed into the finest tracheoles.

The procedure is as follows. Inject as described above. Wash off the adherent solution as rapidly as possible by shaking in several changes of white spirit. Transfer to white spirit saturated with H_2S and bubble H_2S gas through the mixture for 5 minutes to 1 hour depending on the size of the insect. In the case of hard-shelled impermeable parts, such as the thorax of large Diptera, it is necessary to puncture the cuticle first. After gassing, the insects are blotted and fixed in Carnoy's fluid. They can then be mounted whole, dissected, or embedded and sectioned and mounted in Canada balsam.

Technical Details and Results

With the use of cobalt naphthenate very good general injections of the tracheal system in translucent insects can be obtained. For example, in *Campodea*, *Lepisma* (with the scales removed, Pl. I, fig. 4), *Haematopinus*, *Xenopsylla* (Pl. I, fig. 2, shows the general arrangement of the tracheae, and Pl. I, fig. 3, the detail of the elongated air-sacs in the posterior tibia and basal segment of the tarsus), *Cimex* (Pl. I, fig. 5, shows the rich supply of tracheoles to the sucking muscles of the pharynx in the second instar nymph) and the larva and pupa (Pl. I, fig. 6) of *Aedes*. Pl. I, fig. 9, shows a dorsal view of the posterior half of the thorax in *Drosophila melanogaster* when newly emerged, before the

thoracic air-sacs are expanded. The fine tracheal branches can be seen running to the flight muscles from the unexpanded air-sacs. Pl. I, fig. 7, shows a side view of *Drosophila* with fully inflated air-sacs; and Pl. I, fig. 11, a posterior view of the air-sacs and tracheae in the head. Photographs, of course, can show the tracheal vessels in one plane only; they give a most inadequate picture of the actual specimens.

If a living insect is immersed in oil, droplets of water soon begin to exude from the cuticle, particularly when this is thin, as in the intersegmental membranes, or if a light oil such as xylene or white spirit is employed (Wigglesworth, 1942). This same phenomenon occurs in the tracheae and is revealed by the column of injected fluid becoming broken up into alternate black (oily) and colourless (watery) segments. When this happens it detracts greatly from the appearance and usefulness of the preparations.

It follows, therefore, that the precipitation of the sulphide should be effected within the shortest possible time after injection. The cuticle is far more permeable to hydrogen sulphide in the newly moulted insect, and it is very evident in *Drosophila* that more rapid precipitation and, in consequence, more even blackening is obtained in newly moulted than in old insects. The beautifully even coloration of the fine tracheae in the wing pad of the cockroach nymph shown in Pl. II, fig. 4, was obtained in a newly moulted insect. In injecting the rectal papillae of *Calliphora* (Pl. I, fig. 8) it was found desirable to cut off the tip of the abdomen as close to these structures as possible, immediately after injection, and to immerse the exposed tissues at once in white spirit saturated with sulphuretted hydrogen.

Pl. I, fig. 10, is an example of a thick section of the thorax of *Drosophila* after injection and shows the relations of the air-sacs. These sections are 75–100μ thick. A dozen will include the entire insect and make possible the detailed study of the distribution of the tracheae. Miss J. Thomas of this Department is using this method in a study of the comparative morphology of the tracheal system in Diptera. The method is also being used for a study of the tracheal supply of the flight muscles. Pl. II, fig. 3, shows the rich network of tracheoles anastomosing around the fibril bundles in the indirect flight muscles of *Drosophila*, and Pl. II, fig. 2, is a transverse section through these same muscles showing how occasional tracheoles enter the bundles and run a longitudinal course among the fibrils, appearing in cross section as black dots.

Pl. II, fig. 1, shows a longitudinal section through a rectal papilla of *Calliphora*. In the specimen it is possible to follow the course of the intracellular tracheoles very readily and to see, among other things, that most of the fine branches in the cortical epithelium form closed anastomosing loops (cf. Graham-Smith, 1934).

In the course of a study of digestion in *Glossina* (Wigglesworth, 1929, 1931) it was found that during fixation the tracheoles of the gut-wall might become injected with haematin. It was believed that many of these tracheoles were intracellular; and this contention was supposedly confirmed in *Calliphora*.

But the present injection method shows clearly that the tracheoles of the midgut in *Calliphora* are confined to the perivisceral membrane in which they form an immensely rich tangled plexus. At many points this membrane with its tracheoles is invaginated deeply between the cells and the tracheoles may then sometimes have the appearance of being intracellular. Pl. II, fig. 5, is an example of a stained section of the brain of *Drosophila* (10μ thick, stained with Mann's methyl-blue/eosin) showing injected tracheae and tracheoles.

Pl. II, fig. 4, shows a preparation of the wing pad of a half-grown nymph of *Periplaneta* and illustrates the value of the method for making permanent preparations of wing tracheation. Dr. J. Smart of this Department is using the method for studies on the wing venation in Orthoptera.

Methods of Bleaching Injected Insects

The specimen shown in Pl. II, fig. 4, was prepared from a newly moulted cockroach nymph, which has a colourless as well as a readily permeable cuticle. It was desirable, however, to find methods of bleaching dark coloured insects after injection.

On bleaching with hydrogen peroxide or chlorodioxyacetic acid ('diaphanol'), the sulphides of copper and cobalt are oxidized to the sulphates and dissolved. Insects can be bleached slowly by reduction if immersed in 1 per cent. sodium hydroxide saturated with sodium hydrosulphite ($Na_2S_2O_4$) and the metallic sulphides remain unchanged. On exposure to the air the cuticle turns black again; but this can be prevented by adding a drop or two of thioglycollic acid to the reagents used for dehydrating and clearing.

There is, however, a more satisfactory method of bleaching. After fixation in the usual way, the insect injected with cobalt sulphide is soaked well in water saturated with potassium ferricyanide, care being taken that at no stage is the specimen handled with forceps or steel needles. It is then transferred to diaphanol or to hydrogen peroxide, containing potassium ferricyanide. As the insect is bleached the cobalt, instead of dissolving, is precipitated as the deep purple ferricyanide. Pl. II, figs. 6 and 7, show preparations of the head, thorax, and abdomen in *Drosophila* bleached in this way, in which the air-sacs and the main tracheae are well revealed.

An alternative method consists in the use of lead naphthenate. A sample containing 24 per cent. of metal was used. This is a thick brown syrup which gives a solution sufficiently mobile for injection if one-third of its volume of white spirit is added. But better results were given with 50 or 33 per cent. solutions in white spirit or in a somewhat heavier oil (Shell Co. Oil A 12, a very thin lubricating oil in the boiling-point range 265–365° C.). After precipitation of the lead sulphide and fixation as before, the insect is bleached in hydrogen peroxide or diaphanol. The sulphide is oxidized to the insoluble lead sulphate. The insect may then be gassed again with sulphuretted hydrogen and it is reconverted to the sulphide; or, alternatively, the insect may be dehydrated and cleared and mounted in Canada balsam. When examined against a black background the air-sacs and tracheae then show up as opaque

silvery-white structures. It is convenient to mount these cleared insects in Canada balsam in a glass tube of appropriate diameter; they can then be rotated and examined from any desired angle, the tube being mounted in cedarwood oil under a coverslip if necessary. The smaller tracheal branches will not withstand this treatment; but very good preparations of the air-sacs in the head and thorax of *Drosophila* (Pl. II, fig. 8), *Muscina*, and *Calliphora* have been obtained.

Many other modifications of this method can be devised to meet particular requirements. The examples described in this paper give some indication of its range of usefulness.

I am indebted to Messrs. Boake, Roberts & Co., London, for the supply of samples of the naphthenates of copper, lead, and cobalt.

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EXPLANATION OF PLATES

Plate I

Fig. 1. Femur and tibia of *Blattella* × 20 (copper sulphide from copper oleate).
Fig. 2. *Xenopsylla* × 20 (cobalt sulphide from cobalt naphthenate).
Fig. 3. Part of hind legs of *Xenopsylla* showing air-sacs and fine tracheal branches × 50 (cobalt sulphide).
Fig. 4. *Lepisma*, dorsal view of head and anterior segments × 20 (cobalt sulphide).
Fig. 5. Second instar nymph of *Cimex* × 50, showing rich tracheole supply to pharynx (cobalt sulphide).
Fig. 6. Head and thorax of pupa of *Aedes* × 15 (cobalt sulphide).
Fig. 7. Mature *Drosophila* with fully expanded air-sacs in thorax × 21 (cobalt sulphide).
Fig. 8. Whole mount of isolated rectal gland of *Calliphora* × 40 (cobalt sulphide).
Fig. 9. Dorsal view of posterior half of thorax of newly emerged *Drosophila* adult × 110, showing fine tracheae running from the unexpanded air-sacs to the longitudinal flight muscles (cobalt sulphide).

Fig. 10. Transverse section, 75 μ thick, through thorax of *Drosophila* adult at level of the halteres, showing injected air-sacs × 45 (cobalt sulphide).

Fig. 11. Posterior view of head of *Drosophila* showing injected air-sacs and tracheae × 30 (cobalt sulphide).

Plate II

Fig. 1. Longitudinal section through rectal gland of *Calliphora* cut at 30 μ showing medullary and cortical tracheae and intracellular tracheoles arising from the latter, × 150 (cobalt sulphide).

Fig. 2. Transverse section cut at 75 μ through the indirect flight muscles of *Drosophila* × 180, showing tracheoles surrounding the fibril bundles, and occasionally running a longitudinal course within the bundles, where they appear as black dots (cobalt sulphide).

Fig. 3. Side view of indirect flight muscles of *Drosophila* × 250, showing tracheoles anastomosing around the fibril bundles (cobalt sulphide).

Fig. 4. Wing pad of half-grown nymph of *Periplaneta* × 20 (cobalt sulphide).

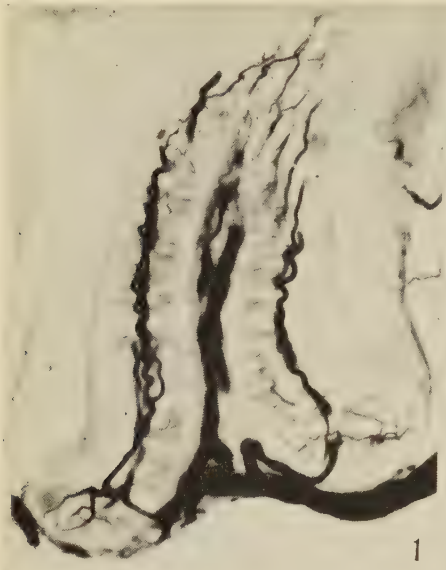
Fig. 5. Section cut at 10 μ through the brain of *Drosophila* × 260 (cobalt sulphide; stained with Mann's methyl-blue/eosin).

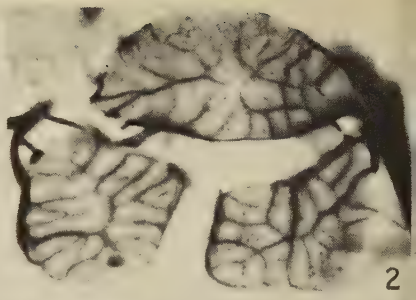
Fig. 6. Oblique view of head and thorax of *Drosophila* × 27, showing air-sacs (cobalt ferricyanide; bleached with diaphanol).

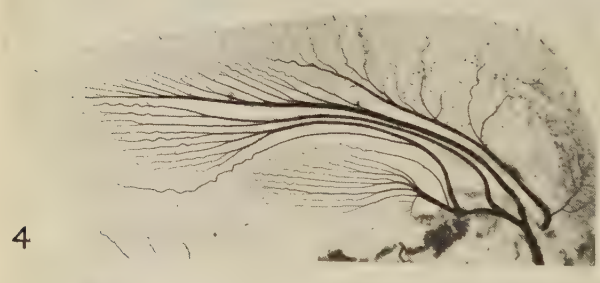
Fig. 7. Oblique view of *Drosophila* × 22, showing thoracic air-sacs and abdominal tracheae (cobalt ferricyanide; bleached with hydrogen peroxide).

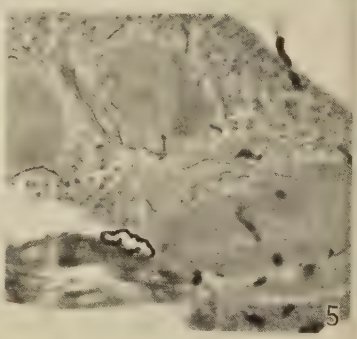
Fig. 8. Side view of *Drosophila* × 30, under dark ground illumination, showing air-sacs in head and thorax (lead sulphate; bleached with diaphanol).

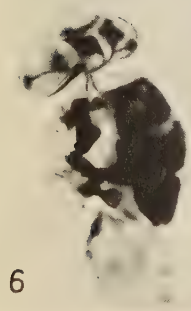
V. B. WIGGLESWORTH—PLATE I

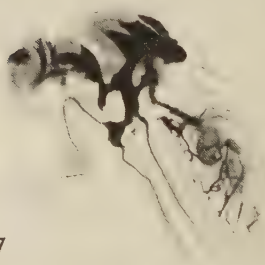












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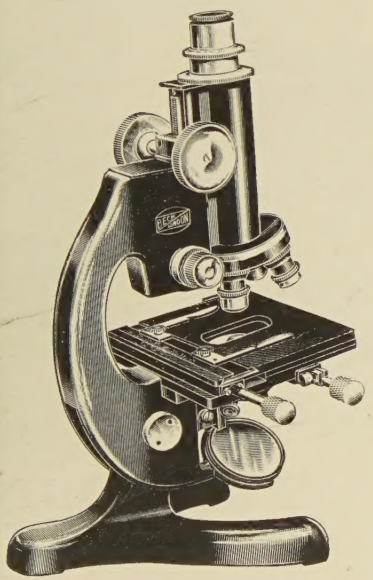

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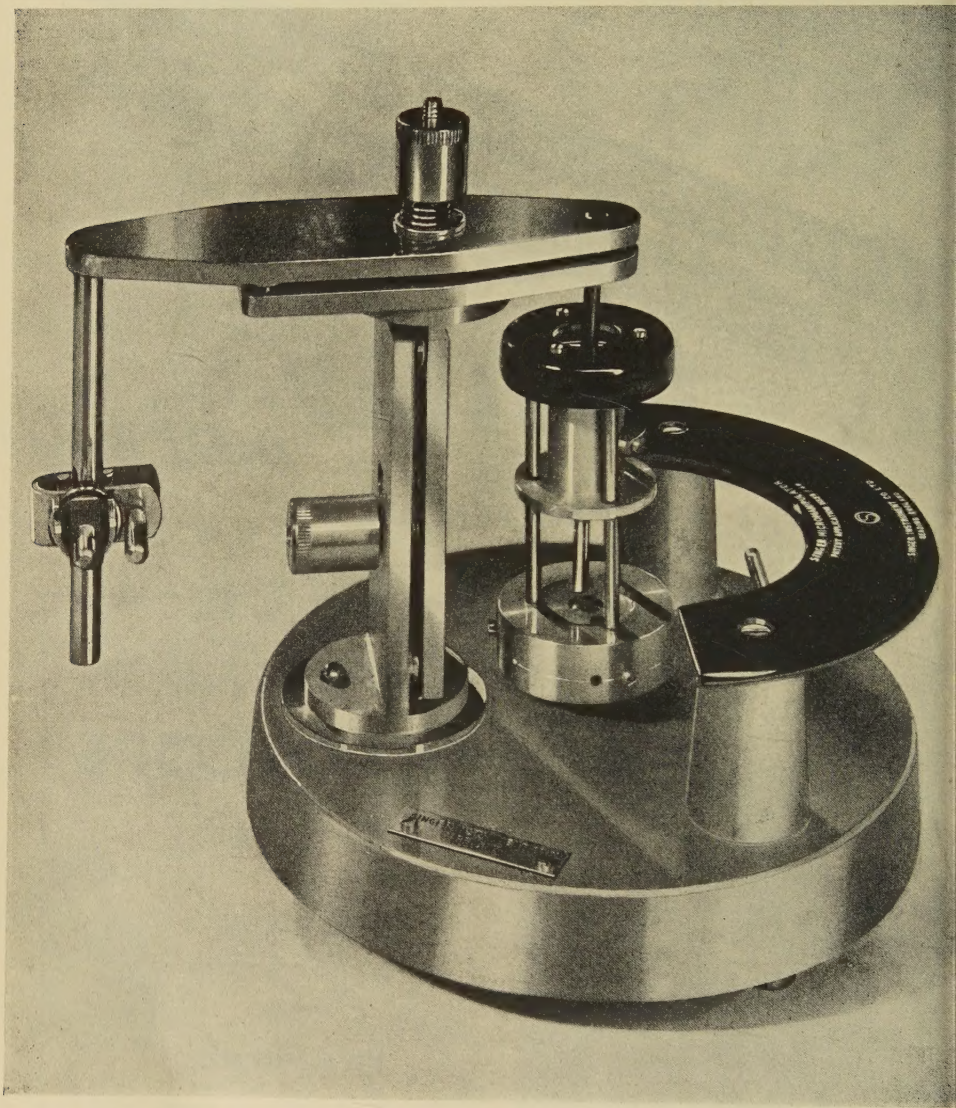

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